Pharmaceutical Bioequivalence Studies: Ensuring Safety, Effectiveness and High Quality

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by

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To my parents, wife, all my children and to the Saudi Government

Statement of originality

This is entirely my own work and all the quotations, illustrations and source materials have been appropriately acknowledged.

Badr A. Aljohani (2nd September 2015)

Abstract

Poor quality medicines are a global problem affecting both developed and developing countries. Governments and the health authorities are focusing on the spread of counterfeit medicines, as it is a threat to patients and funds criminal activities.

Recently questions have been raised about using generic substitutes, especially for Narrow Therapeutic Index Drugs (NTIDs), such as ciclosporin. *In-vitro* dissolution testing was undertaken to identify differences in dissolution performance between branded and generic ciclosporin capsules. Dissolution testing of the capsules was carried out according to the USP guidelines. According to the USP not less than 80% of the labelled amount of ciclosporin should dissolve in 90 min. The samples were analysed using a HPLC method.

Two ciclosporin generic products showed less than the minimum percentage of labelled amount $< 80\%$. Statistical analysis showed significant differences ($p<0.0001$) of the mean percentage content between brand and generic. Investigations were carried out to detect impurities in ciclosporin capsules using LC-MS. Concentrations of inactive ingredients such as sorbitol were variable between capsules. One from South America, manufactured in central Asia, showed contamination with a plant product (Zizyphine A). the synthetic intermediate (Delcorine) was found to be more than 1000 fold higher in the generic product compared to reference capsules ($p<0.001$).

In 2013, the FDA warned of the possible fatal effect of azithromycin. LC-MS quantification for azithromycin tablets were carried out in order to quantify azithromycin content in different products. A bioequivalence study in man, confirmed that generic (Mazit) capsules were bioequivalent with brand (Zithromax™) capsules.

Based on the results presented in this thesis, HPLC and LC-MS proved suitable approaches for analysis of drugs and their unknown impurities in brand, generic and counterfeit medicines. Some ciclosporin preparations did not contain the mass labelled. Therefore, switching between branded and generic ciclosporin may lead to undesirable effect.

Publications

Abstracts:

1. Aljohani B, Al Otaibi F, Ghazaly E, Perrett D, Johnston A. December 2011. Development and validation of a h.p.l.c. method for ciclosporin: its application to measurement of brand and generic versions from different countries. *British Journal of Clinical Pharmacology, 73(6), 1007.*
2. Aljohani, B., Al Otaibi, F., Ghazaly, E., Perrett, D., Johnston, A. December 2012. Tracking the counterfeit and substandard of ciclosporin capsules by high performance liquid chromatography. *Proceedings of the British Pharmacological Society at* *http://www.pA2online.org/abstracts/Vol10Issue4abst117P.pdf*
3. Aljohani, B., Ghazaly, E., Perrett, D. & Johnston, A. 2014. Analytical techniques for tracking counterfeit and substandard medicines. J Bioequiv Availab, 6, 48.
4. Aljohani, B., Ghazaly, E., Perrett, D. & Johnston, A. 2014. Impurities detection in ciclosporin capsules: a comparison with Neoral®. Proceedings of the British Pharmacological Society at *http://www.pa2online.org/Vol1Issue1abst001P.html*, 1, 001.

Oral presentations:

1. Aljohani, B., October 3, 2012. Tracking Counterfeit and Substandard Medicines by Capillary Electrophoresis. CE in Biotechnology & Pharmaceutical Industries: 14th Symposium on the Practical Applications for the Analysis of Proteins, Nucleotides and Small Molecules, Scottsdale, Arizona, United States.
2. Aljohani, B., 2013. From package to the patient, is it the right medication? 20th International Reid Bioanalytical Forum. Guildford, United Kingdom: The Chromatographic Society.

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List of abbreviations

AUC	Area under the plasma concentration-time curve
AE	Adverse events
ANOVA	Analysis of variance
Brand E	Brand ciclosporin from Egypt
Brand Egy	Brand ciclosporin from Egypt
Brand J	Brand ciclosporin from Jordan
Brand Jor	Brand ciclosporin from Jordan
Brand P	Brand ciclosporin from Pakistan
Brand Pak	Brand ciclosporin from Pakistan
Brand S	Brand ciclosporin from Saudi Arabia
Brans Sa	Brand ciclosporin from Saudi Arabia
Brand T	Brand ciclosporin from Turkey
Brand TK	Brand ciclosporin from Turkey
BPAR	Biopsy proven acute rejection
C2	Single point measurement of plasma ciclosporin after 2 h

CI	Confidence interval
C_{max}	Maximum drug concentration
Conc.	Concentration
CRF	Case report form
CyA	Ciclosporin A
CycA	Cyclophilin A
CV	Coefficient of variation
CYP3A4	Cytochrome P3A4
CYP450	Cytochrome P450
ECG	Electrocardiogram
EMA	European Medicines Agency
ESI	Electrospray ionization
FDA	Food and Drug Administration
Generic C	Generic ciclosporin from Colombia
Generic Col	Generic ciclosporin from Colombia
Generic I	Generic ciclosporin from India

Generic M	Generic ciclosporin from Morocco
GLP	Good laboratory practice
GMP	Good manufacturing practice
GI	Gastrointestinal
h	Hour
HPLC	High performance liquid chromatography
HMDB	Human metabolome database
HSS	High strength silica
ICH	International conference on harmonisation
KEGG	Kyoto encyclopaedia of genes and genomes
Kg	Kilogram
LC-MS	Liquid chromatography mass spectrometry
L	Litre
mg	Milligram
min	minutes
µg	Microgram

MHRA	The UK Medicines and Healthcare products Regulatory Agency
mL	Millilitre
mTorr	millitorr
MS	Mass spectrometry
mV.min	Millivolt per Minute
mV.s	Millivolt per Second
MW	Molecular weight
Neg.	Negative
NF	National Formulary
NTID	Narrow therapeutic index drug
PDE	Permitted daily exposure
Pos.	Positive
ppm	Parts per million
QT interval	Time between Q wave and T wave in electrical cycle of the heart
RPLC	Reversed phase liquid chromatography
SD	Standard deviation

SMS	Short message service
S.R.L	Italian abbreviation for limited liability company, (Società a responsabilità limitata)
$t_{1/2}$	Plasma elimination half life
TDM	Therapeutic drug monitoring
TGA	Therapeutic goods administration
t_{max}	Time to maximum drug concentration
TSQ	Triple-stage-quadrupole
UK	United Kingdom
UHPLC	Ultra high performance liquid chromatography
US	United States
USP	United States pharmacopeia
UV	Ultraviolet
UV/Vis	Ultra/visible
WHO	World Health Organisation

Chapter 1. Introduction

1.1 The nature of the problem

Substandard and counterfeit medicines are considered to be a worldwide problem, affecting both developed and developing countries. The toughest realization is that there are many cases where counterfeit drugs have caused serious harm to consumers, including death. These are serious consequences and add a real urgency in the fight against counterfeit drugs. The consequences are well known, as the Department of Homeland Security (DHS) advised, counterfeit and substandard medicines could cause a serious threat to America's economic vitality, the health and safety of American consumers, and our critical infrastructure and national security (U.S. Customs and Border Protection Office of International Trade, 2012).

Many incidents as a result of using such drugs, have been reported. In Nigeria, 2500 deaths were reported in 1995 because of fake vaccines. In 1995 and 1998, 89 deaths in Haiti and 30 deaths in India occurred because of using a paracetamol preparation that contained di-ethylene glycol. In 1999, 30 people died in Cambodia after using a counterfeit antimalarial preparation prepared with sulphadoxine-pyrimethamine (World Health Organization, 2006). Developing countries are a clear target for counterfeiters, because of the high prices for genuine medications and weak supervision from the authorities, but developed countries could be a target too. In 2006, UK authorities detained around £500,000 worth of anti-flu medication that was counterfeit (Mukhopadhyay, 2007). In 2012, the Food and Drug Administration (FDA) announced that a fake anti-cancer drug had been found in US clinics (Figure 1.1). It was confirmed that the fake intravenous 400 mg/16 mL Altuzan® (bevacizumab) had no active ingredients (Food and Drug Administration, 2012a). In 2002 Amgen pharmaceuticals issued a warning about counterfeit Epogen® (an anti-anaemic drug) which had been intentionally relabelled with a higher dosage to sell it at a higher price (Amgen Inc., 2002, C. and Tigue, 2006). Another example of critical medication being counterfeited is a case in which heparin, an anticoagulant drug had its active ingredient substituted for a cheaper alternative. This unfortunately resulted in the suspected deaths of 81 patients. A nationwide recall of heparin was announced (Food and Drug

Administration, 2008c). Further investigation carried out by the FDA confirmed that the active ingredient, which was the main contaminant, originated out of 12 Chinese companies and had made its way into 11 countries (Food and Drug Administration, 2008b).

Figure 1.1: Fake anti-cancer drug found in a clinic, USA

Counterfeit drugs in the United States are growing rapidly, in particularly due to ever increasing suppliers from around the world (Blackstone et al., 2014). In 2006, the World Health Organization estimated that the business in counterfeit drugs could be worth US $75 billion globally by 2010. The size of the problem today is likely to be even greater than these estimates. Dr Lee Jong-wook, General Director of WHO said "Combating low quality or illegal medicines is now more important than ever" (Pincock, 2003).

In 2011, a TV report on the Al-Arabiya News Channel highlighted the problem of the smugglers and counterfeiters of medicines in Yemen. Millions of patients seeking a cure fell victims of expired smuggled, forged and substandard medications. Health authorities discovered that some lifesaving medications, e.g.: anti-D (Rho) immunoglobulin and vaccines, were only water. Health professionals in Yemen blamed the government, agents and health authorities for lack of supervision. They called it “Trade of Death”. Many reasons for smuggling and faking include drug availability, no alternative, and high prices.

By the year 2000, 80% of medications in the market were smuggled with the majority containing low or no active ingredients (Al Arabiya News Channel, 2011b). Another TV report focused on fake medicines in West Africa. Fake medicines were manufactured in Pakistan, India and China and imported to developing countries. This resulted in deaths and blindness in patients. Reasons for the increase of the fake and substandard medicines were, lack of government supervision and poor storage conditions. Some medications were sold on the street with half price for expired medications (Al Arabiya News Channel, 2014).

In May 2014, Interpol reported that during a single week, police/customs in 111 countries seized 9.4 million doses (2.4 million in 2011) of fake medicines including cancer medication, erectile dysfunction pills, anti-malarial and cholesterol medications, worth nearly USD 36 million (Al Arabiya News Channel, 2011a, Interpol, 2014).

Recently, the WHO announced a drug alert for counterfeit meningitis vaccines circulating in West Africa (Figures 1.2 and 1.3). WHO advised increased supervision of the medications, further verification and checks with drug manufacturers should be made before administration (World Health Organization, 2015).

Figure 1.2: Falsified version of meningitis vaccines, West Africa

Figure 1.3: Another falsified version of meningitis vaccines, West Africa

Innovators, whose key activities are to create or obtain the rights or exclusivity to their work, make use of intellectual property rights and registration. These rights are appointed in the form of trademarks, copyrights and in the case of the pharmaceutical industry, patents. These ensure that all innovations and efforts are protected to a certain degree within the legitimate marketplace. As sufficient as these measures are within the pharmaceutical industry, it is undermined by theft associated with counterfeiting and trafficking of pharmaceuticals products (Blackstone et al., 2014).

Counterfeiting may be related to organised crime across the world, assisting the likes of money laundering and terrorism (Pfizer Inc., 2007). Profits made could well exceed those compared with narcotics. Drug companies are investing their efforts to fight these criminal acts by applying new technologies, such as short message service (SMS) authentication code systems on packaging. They are working in co-operation with health authorities across the world like the British Medicines and Healthcare products Regulatory Agency (MHRA), the Australian Therapeutic Goods Administration (TGA), and the FDA to fight against these crimes.

Distribution channels for pharmaceuticals in the European Union is going through a major regulatory update. A new directive was adopted in July 2011 to protect patients and to prevent the distribution of fake medicines (European Medicine Agency, 2012). These new directives applied from January 2013. However, for effective implementation for the new directives, it may take up to 3 years (Williams, 2011).

1.2 Substandard and counterfeit medicines

Substandard medicines do not contain the correct amount of active ingredients or meet the standard (innovator's) preparation requirements for quality, safety and efficacy (European Medicine Agency, 2012, Food and Drug Administration, 2012b).

According to the FDA, counterfeit medicine is "fake medicine. It may be contaminated or contain the wrong or no active ingredient. They could have the right active ingredient but at the wrong dose". They may have the wrong manufacturer's name or the country of origin. Counterfeits may include both branded and generic products. Using such drugs may cause harmful effects to patients (Figure 1.4).

Substandard and counterfeit drugs can make their way into the market place. This can occur in both branded and generic drugs. Higher or lower levels of the active ingredient which differ from the required formulation are regarded as a substandard drug (Johnston and Holt, 2014).

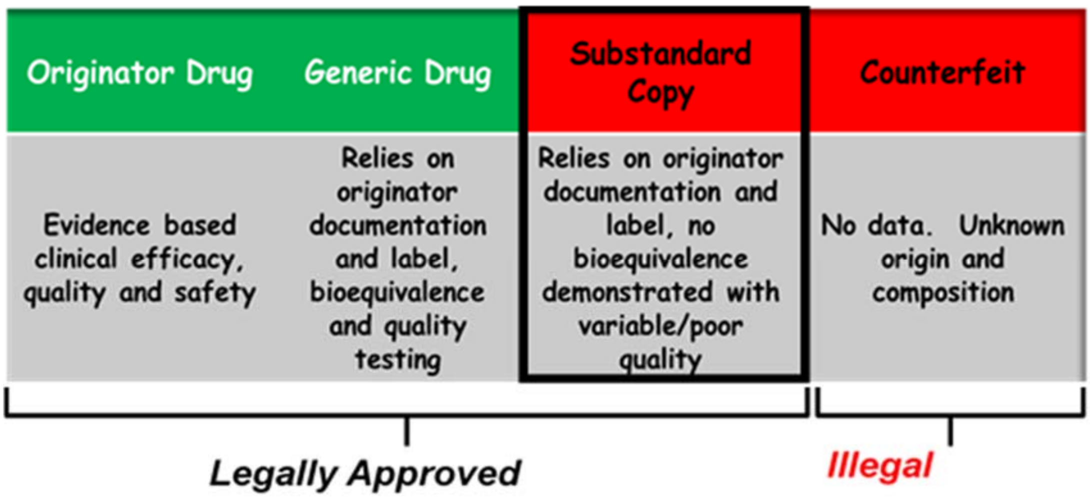

Figure 1.4: Definitions of brand, generic, substandard and counterfeit medicines

There are several effects that consumers could experience when using substandard and counterfeit drugs. This all varies on which element of the drug fails to meet the required quality levels. With low quality antibiotics, the patient's treatment plan can be highly unsuccessful and face additional risk of adverse effects. With substandard and counterfeit antibiotics, this can increase the level of antibiotic resistance and there is loss of confidence in the treatment method by both physicians and patients. Some other scenarios include where the non-active ingredients do not meet the required limits and/or contamination of the drug (Kelesidis et al., 2007, Kelesidis and Falagas, 2015).

1.3 Factors affecting the spread of counterfeit and substandard medicines

There are many factors encouraging the spread of counterfeit and substandard medicines. These factors include poor regulation in countries that do not establish or have good regulatory mechanisms and weak supervision from authorities. The profit that can be made by selling fake medicines is one of the reasons. According to Blackstone et al., counterfeit medicines are more profitable than selling narcotics (Blackstone et al., 2014). Drug availability is an important reason as well, because if the drug is not available on the market patients will search for other available resources. Low supply levels are a reason why counterfeit drugs enter the supply chain in the United States. Some drugs, which are considered lifesaving, such as anti-cancer drugs, are in high demand and offer opportunist counterfeiters the chance to supply a high demand drug at higher than normal prices. But as these counterfeit lifesaving drugs are sold to consumers, there is greater risk of suffering and even death to patients.

The lack of regulation, especially in free trade zones, is considered an important reason for the prevalence of fake medications. In addition, the packaging and storage conditions may affect the quality of the drug if it is not stored under proper conditions. A study of generic ramipril tablets showed that 24% out of 17 samples failed to meet the drug quality requirements because of improper storage conditions (Khan et al., 2013, Johnston and Holt, 2014). These problems are present in all global regions, but particularly affect most developing countries. According to the FDA, 10% of medications in both industrialised and developing countries are counterfeit. In rich countries, expensive medicines used for body building, such as hormones and steroids, are subject to higher levels of counterfeiting (World Health Organization, 2006).

Even in a country such as the United Kingdom with strict regulations, there is infiltration of counterfeit drugs because of loopholes in transit and supply chains. The common vulnerabilities in the supply chain are due to the many handling levels associated in reaching the end consumer. Because of the many points of entry into the supply chain, detecting substandard or counterfeit products poses a much bigger challenge. Unfortunately, substandard and counterfeit drugs make their way into legitimate supply chains and these also include reputable online pharmacies. There are many points of entry for counterfeiters to enter during the supply chain from key ingredients to manufacturers, to storage vulnerabilities, transportation and several points at the final distribution channels. 90% of drugs distributed in the United States are handled by national wholesalers who deliver direct to pharmacies, hospitals, clinics and physicians. The remaining 10% unfortunately have a more complicated and indirect method of distribution and due to flaws in their practices, give a greater chance for counterfeits to enter the supply chain. Further regulation and monitoring need to be successfully put in place to avoid such vulnerabilities. The wholesale market comprises of three types, the primary wholesalers which typically deal directly with the manufacturers and pose the least risk for substandard and counterfeiting to occur. Secondary wholesalers are comprised of large regional wholesalers which can package and repackage. The third comprises of thousands of smaller wholesaler (Yadav, 2014).

The internet is the main source of purchasing medications in most of Europe and it was recently revealed that out of a survey of 3,100 online pharmacies, only four were certified through the verified Internet Pharmacy Practice (American Enterprise Institute For Public Policy Research, 2012). Many online pharmacies are considered a real threat to consumers because they offer potentially harmful medications. High profits can be made selling substandard and counterfeit medications, especially without the need for a prescription. Some patients may prefer an online pharmacy as it can provide an additional level of privacy as some conditions they may find embarrassing, such as impotence. For disabled patients it is also more convenient as they may be home bound (Orizio et al., 2010). Ordering online medications consisted more of lifestyle drugs such as Viagra but there is an increase in the use of prescribed medications via the Internet for more serious diseases, such as cardiovascular, diabetes and cancer. A higher threat of using online pharmacies for purchasing NTID such as ciclosporin, patients could be at risk of either toxicity at higher doses or treatment failure with lower doses. Examples of NTIDs which have been found to be available from online pharmacies (Liang et al., 2013):

- Aminoglycosides
- Amikacin
- Gentamicin
- Rifampicin
- Warfarin
- Ciclosporin
- Carbamazepine
- Lithium
- Phenytoin
- Theophylline
- Phenobarbital
- Valproic acid
- Digoxin

A death was reported due to consumption of a drug which was ordered online. A teenager took one pill to treat his anxiety disorder and this resulted in his death (Broomhead, 2014). Many counterfeit online pharmacies are falsely stating that they are based and operate from Canada, as Canada is considered the 9th largest country in pharmaceutical sales, with a global share of 2.5% (Jackson, 2015). In 2005, a study revealed that out of 11,000 online pharmacies registered to be based in Canada, only 214 were legitimate (Blackstone et al., 2014).

1.4 Methods for detecting counterfeit medicines

The war against counterfeit medicines should be united worldwide. There should be a common definition for counterfeit medicines and a universal law against it. The UK government takes many actions against counterfeit drugs. Supplying or sale of counterfeit medicines is considered a criminal offence.

There are many tests carried out to check for counterfeit drugs. Package and general characteristics (size, shape and colour of the drug) analysis is a key step for detecting counterfeits. If there are any changes or doubts raised from these initial examinations, they should be investigated by the pharmacist and reported so that appropriate action can be taken. For further investigation chemical analysis is considered. Spectroscopy analysis such as Fourier-transform infrared, near infrared and Raman spectroscopy can establish the identity of most drugs and discriminate from closely related or structurally similar compounds (Moffat, 2008). Other detection methods use microscopy techniques, such as light microscopy and the scanning electron microscope. Separation techniques are used to detect counterfeits such as liquid chromatography, capillary electrophoresis, and mass spectrometry (Moffat, 2008). HPLC methods with separation are generally an acceptable technique. This is because HPLC methods can generally provide the required specificity. All drug products should have a specific assay to determine the content of the drug.

In 2011, Pfizer Inc. started implementing their SMS authentication code system on packaging. Patients and consumers can send an SMS containing the pack code to the company and receive verification of the product's authenticity (Marsh, 2011).

On November 28, 2013, President Obama signed the Drug Quality and Security Act into law, which provides for a national track-and-trace system that would allow a specific drug to be followed from the manufacturer to the pharmacy. This should make it more difficult for counterfeiters to enter the supply chain in the United States. This law is expected to be implemented in 2015 (Food and Drug Administration, 2013a).

Specific tests should be developed on an individual product basis. The dissolution test gives an idea of the release of the drug substance from the drug product when taken orally. Although one time point measurement is enough for immediate release drugs, suitable test conditions and sampling procedures should be established and reported for non-immediate release drugs. If a drug is not available in the required time after it has been taken orally, it will significantly affect the bioavailability (rate and extent of the drug inside the body). Drug disintegration, tablet hardness, water content and microbial limits should be thoroughly investigated, reported, and documented to identify counterfeit and substandard medicines.

1.5 Safety of drug use: branded versus generic

The branded drug is a new medication that is found to improve or treat a certain disease or medical problem. This new medication passes through many phases of developmental and research processes. The phases include the preclinical testing in animals for pharmacokinetic and pharmacodynamics of the tested drug, phase I studies which are conducted on 20 – 80 healthy volunteers for safety screening, phase II studies to ensure the effectiveness of the drug, phase III studies to get more information about the safety and effectiveness (subjects range from 200 – 3000 people) and phase IV which consists of the post approval studies (Figure 1.5). These phases can happen over many years. The product can only be in the market once it has been approved by a regulatory health body such as the FDA. The manufacturer usually gets a patent for the new drug for a period of up to 20 years (Pipeline patent intelligence, 2011). Patenting the product prevents other companies from making and selling the new product. When the patent period has expired, other companies can start making and selling a generic version, but it must be evaluated and approved by the FDA or other regulatory health authorities. Over the last few years, the production of generic medications has increased rapidly due to competition after expiry of the originator drug company's patent (Food and Drug Administration, 2009c).

Figure 1.5: General phases for new drug development. Adapted from innovation.org

The FDA approved an average of 101 new generic drugs each year from 2001 until 2014 into the market place (Figure 1.6). These included many drug classes: hormones, antibiotics, analgesics, cardiovascular, respiratory, antimetabolite and many other classes (Food and Drug Administration, 2015a).

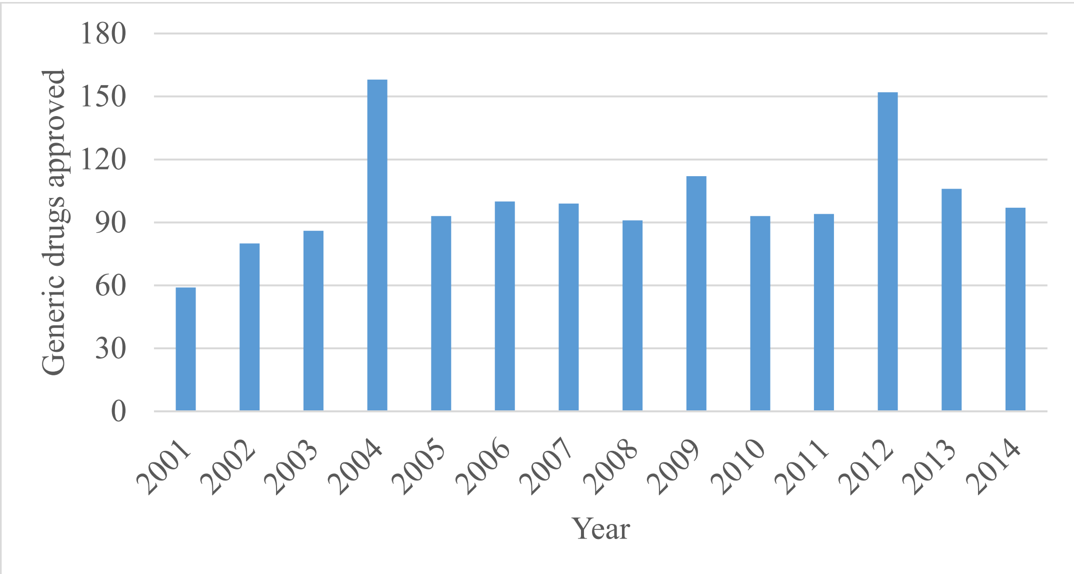

Figure 1.6: FDA generic drug approvals from year 2001 until 2014

Due to competitive market demands, many generic drug companies place their products on the market as soon as the patent period of the brand medicine has expired bringing concerns for patients safety because in some cases they are not safe and effective like their reference counterpart. Some tentative applications may even be approved before the patency has even expired. There have been many reported clinical studies highlighting the adverse effects of generic products (Gautam et al., 2009, Newton et al., 2010, Perks, 2011). During recent years, the production of generic medications has increased due to demand for cheaper medicines especially in developing countries. The generic medications may have lower therapeutic effects and/or toxic effects (Newton et al., 2010). Due to the lack of good manufacturing practice, insufficient bioequivalence and toxicological studies (Gautam et al., 2009), and even deliberate forgery of the drug, there are an increasing number of patient safety concerns. Small differences in plasma concentrations, less than 4%, may exist in some cases between a brand and its generic equivalent. However, this minor difference is no greater than the difference that may exist between two different manufactured batches of the brand drug manufactured by the same pharmaceutical company. Safety and efficacy trials are only required for new drugs (Nation and Sansom, 1994). Therefore the average cost for the production of generic drugs is lower than the brand by approximately 45% (Canadian Health Services Research Foundation, 2007).

Bioequivalence is the equivalence of the action of brand and generic medicines. Birkett, described bioequivalence as: "Two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent and their bioavailabilities (rate and extent of availability) after administration in the same molar dose are similar to such a degree that their effects, with respect to both efficacy and safety, can be expected to be essentially the same. Pharmaceutical equivalence implies the same amount of the same active substance(s), in the same dosage form, for the same route of administration and meeting the same standards" (Birkett, 2003).

A generic drug is bioequivalent when the active ingredients or the active metabolites are absorbed into the body after administration at the same rate and amount as the brand drug. Thus, the need for bioequivalence is evident by the fact that the generic products deliver the same therapeutic effect as the branded counterpart and can safely substitute the brand product. Before a generic drug can be marketed, the manufacturer must prove that it has the same potency and efficacy as the brand medication. If a generic passes these tests, it is said to be bioequivalent to the original drug. The generic drug application goes through several stages before approval by the FDA. Key areas being monitored are chemicals, manufacturing processes, bioequivalence tests, dissolution tests, labelling, etc.

Bioequivalence gives health professionals and patients the confidence that the generic medicines provide the same therapeutic effect, clinical results and safety profile as their branded counterparts. Bioequivalence studies play an important role during drug development to observe the optimum therapeutic effect and ensure no additional toxicities (Birkett, 2003).

According to the FDA guidelines, bioequivalence is accepted when 90% confidence intervals (CI) for the ratio of target pharmacokinetic parameters of area under the curve (AUC) and maximum concentration (C_{max}) fall in the range between 0.8-1.25 (80-125%) (Food and Drug Administration, 2003a). The time to maximum plasma concentration (t_{max}) should also be similar. Bioequivalence studies are an important part of drug development for the production of new drug formulations and for generic equivalents. Such studies are important after the approval phase if there are any manufacturing changes (European Medicines Agency, 2010). Many countries have established guidelines for the approval of generic drugs. Generic manufacturers are not required to submit data of clinical trials or preclinical tests which are long and expensive procedures, but they must submit proof of bioequivalence tests, in addition to other pharmaceutical information (European Medicines Agency, 2008). During bioequivalence studies, some minor differences between brand and generic drugs are allowed. For example, a generic drug may have differences in shape, size or colour compared to the branded product (Kesselheim et al., 2008).

Since generic and therapeutic substitution might impact on the clinical outcomes, it could create a conflict between the interests of patients, clinicians and those of payers/providers (AlAmeri et al., 2010). Patients who are uncertain are warned that substitution that is done only for financial reasons might compromise their quality of care. They believe that a cheaper medicine must be inferior to the more expensive branded medicines (Meredith, 2003).

Many healthcare providers have been promoting generic and therapeutic substitution in an attempt to contain their costs (Duerden and Hughes, 2010). In 2013, it was reported that generic prescribing had reached 83.9% of all prescribed items in community pharmacies in England (Health and Social Care Information Centre, 2014). Furthermore, the Department of Health (DOH) in England considered and then abandoned the idea of automatic generic substitution of medicines by pharmacists (Baker et al., 2009, The Pharmaceutical Journal News team, 2010). Accordingly, pharmacists and other dispensers who receive a prescription containing a branded medicine would be obliged to dispense an equivalent generic version of the medicine instead.

The FDA and the European Medicines Agency (EMA) have set out guidelines to establish the requirement range for bioequivalence for generic drugs, which should be between 80-125% of the original innovator drug's bioavailability (European Medicine Agency, 2001, Food and Drug Administration, 2015b). On the other hand, many authors question the approval of such range limits for NTIDs such as ciclosporin (Bartucci, 1999, Cattaneo et al., 2005, Johnston and Holt, 1999a, Kahan, 1999, Kamerow, 2011, Sabatini et al., 1999). A small dose difference in NTIDs could have serious side effects which could lead to treatment failure and/or toxicity (Johnston and Holt, 2001).

1.6 Excipients in medicines

Excipients are the inactive ingredients in a drug that include binders, fillers, lubricants, sweeteners, preservatives, flavours, colouring, printing inks, etc. (Wandel et al., 2003, Iheanacho and Blythe, 2009). Although excipients are considered to be inactive ingredients that do not have a therapeutic effect, some studies have shown that excipients can cause many side effects (Wandel et al., 2003). Figure 1.7 shows the differences in sizes, shapes and colours of the same medicine.

Excipients do not need to match the innovator's drug formulation. Some evidence shows that different excipients are metabolised differently in the body such as polyoxyethylated castor oil and polysorbate 80 (Johnston and Holt, 2014).

Figure 1.7: Different generics of the same medicine (lisinopril 20mg) showing differences in sizes, shapes and colours

Another example of phenytoin (antiepileptic agent) toxicity happened in Australia because of changing the excipient in phenytoin to lactose instead of calcium sulphate. That change affected the solubility of phenytoin and made it more soluble which increased its systemic availability, which led to an increased incidence of toxicity (Tyrer et al., 1970).

Another example is of a drug company decreasing the particle size of digoxin powder from 20 μm to 3 μm to formulate digoxin tablets. That caused an increase in the drug absorption up to twofold. Consequently, many patients had signs of toxicity (Johnston et al., 1997). Therefore, many authors have highlighted the importance of the bioavailability of pharmaceutical products especially for critical dose and NTID (Johnston et al., 2004, Holt, 1978).

1.7 Impurities in drug products

Impurities may be defined as any ingredients, substances or contaminations which do not belong to the active or non-active ingredients of the drug.

FDA guidelines classify impurities in new drug substances into organic, inorganic or from residual solvent. These impurities may affect the drug product quality and lead to serious adverse effects affecting patients' safety. The origin of these impurities can be from the synthetic procedures for the active ingredient or from the degradation of the inactive ingredients in the drug product (Basak et al., 2007, Roy, 2002).

Organic impurities may arise from degradation of the new drug substance or the manufacturing process. The acceptance limits of these impurities should be well specified. If an impurity in a drug product is coming from different sources such as a synthetic product and also a degradation product, it should be monitored and included in the impurity limits. The medication properties can be changed or could result in toxicity by impurities.

1.8 Organic impurities

Organic impurities can occur through the manufacturing procedures or during the storage of the drug substances and they include the following:

1.8.1 Starting or intermediate materials

Most common impurities come from this source, and are found in each active ingredient unless specific considerations and care are taken through the production. For example in paracetamol bulk, the para-aminophenol content is limited by specific tests, which could be a starting material for one manufacturer or an intermediate for another (Figure 1.8).

1.8.2 By-products

During the production of organic chemicals, it is very uncommon to get a finished product with 100% yield. It is common to have impurities from this source, for example in paracetamol bulk, diacetylated paracetamol (paracetamol with an attached butane-2,3-dione group) may be formed as a by-product (Roy, 2002).

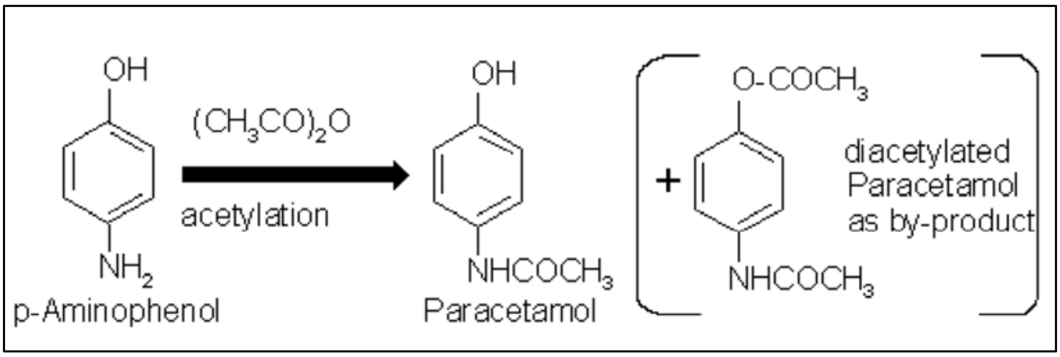

Figure 1.8: By product impurity formation during production of paracetamol from *p*-aminophenol. Adapted from Roy (2002)

1.8.3 Degradation products

Degradation products are yet another source of impurities, which can affect drug quality level during the synthesis. Impurities can also be formed by degradation of the finished product. However, the degradation of the product can occur with storage and/or ageing. The degradation of penicillins and cephalosporins is a well-known example of degradation products. The presence of a ß-lactam ring as well as that of an α-amino group in the side chain plays an important role in their degradation (Figure 1.9 and Figure 1.10).

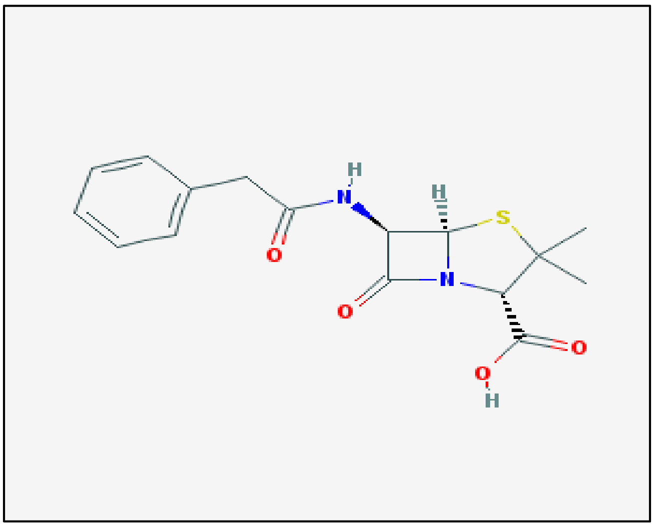

Figure 1.9: General structure of penicillins (National Center for Biotechnology Information, 2004)

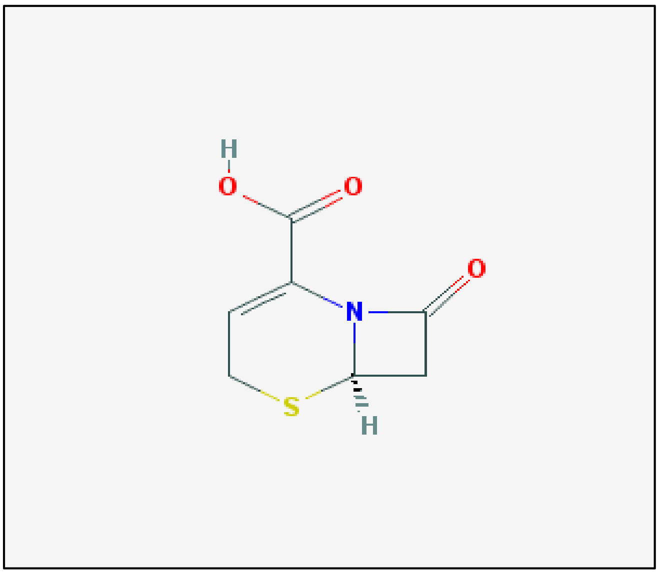

Figure 1.10: General structure of cephalosporins (National Center for Biotechnology Information, 2011)

1.8.4 Reagents, ligands and catalysts

These sources of impurities are rare in active ingredients. However they still should be considered. In general, some active ingredients may contain several sources of organic impurities mentioned above at levels varying from insignificant to critical.

1.9 Inorganic impurities

Inorganic impurities usually come from the production and the manufacturing procedures. They are normally known and identified and include the following:

1.9.1 Reagents, ligands and catalysts

There are limited possibilities for these kinds of impurities to happen. However care must take place during the production otherwise it may result in a product that exceeds permitted limits.

1.9.2 Heavy metals

During the production, the two main sources for heavy metals are water and chemical reactors. They may cause acidification or hydrolysis of acid during manufacturing.

These impurities of heavy metals can be prevented by using demineralised water and glass lined instruments.

1.9.3 Other materials

During the manufacturing process many types of filters may be used. Activated charcoal may be used for filtration. These impurities could be avoided by regular monitoring of fibres and black particles in the product (ICH Harmonised Tripartite Guideline, 2002, Roy, 2002, Food and Drug Administration, 2003c).

1.10 Residual solvent

According to FDA guidelines residual solvents can be defined as organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products.

The solvent is one of the important factors during the manufacturing procedures as residues may be harmful. If it cannot be removed completely during the synthetic process, then the product might not meet specifications, affect good manufacturing practices, or interfere with other quality based requirements. Based on these facts, there should be suitable selection of the solvent for the production of a drug as it may increase the yield, or modify characteristics such as crystal form, purity, and solubility (Roy, 2002, Food and Drug Administration, 2003b).

1.10.1 Solvent classification

According to ICH guidelines, residual solvents are evaluated according to their possible hazard effect to human health and classified under three classes (European Medicines Agency, 2009)

Class 1 solvents: Solvents to be avoided.

Known human carcinogens, strongly suspected human carcinogens, and environmental hazards (Table 1.1).

Table 1.1: Class I: Solvents to be avoided in medications

Solvent	**Concentration limit (ppm)**	**Concern**
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethane	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

*Source: (Food and Drug Administration, 2003b, European Medicine Agency, 2009)

Class 2 solvents: Solvents to be limited.

Non genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity. Solvents suspected of other significant but reversible toxicities (Table 1.2).

Table 1.2: Solvents to be limited in medications

Solvent	Permitted Daily Exposure PDE (mg/day)	Concentration Limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
N,N-Dimethylacetamide	10.9	1090
N,N-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethylene glycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
N-Methylpyrrolidone	48.4	4840
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloroethene	0.8	80
Xylene	21.7	2170

*Source: (Food and Drug Administration, 2003b, European Medicine Agency, 2009)

Class 3 solvents: Solvents with low toxic potential.

Solvents with low toxic potential to man where no health-based exposure limit is needed. Class 3 solvents have permitted daily exposure of 50 mg or more per day (Table 1.3).

Table 1.3: Solvents with low toxic potential*

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl acetate	Methyl-ethyl ketone
Tert-Butylmethyl ether	Methyl-isobutyl ketone
Cumene	2-Methyl-1-propanol
Dimethylsulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	Tetrahydrofuran

*Source: (Food and Drug Administration, 2003b, European Medicine Agency, 2009)

1.11 Other sources of impurities

Impurities in the drugs can also occur as a result of exposure to the heat, light, change in pH, interaction with the package component or with other active ingredients if it is a combination drug (Kovaleski et al., 2007).

1.12 Aims and objectives

In this thesis, the growing issue of substandard and counterfeit medicines and how methods used to reduce these challenges are reviewed. The challenges highlighted clearly affect all developed and developing nations on many levels.

Using generic substitutions has the advantage of a reduced cost to the healthcare system and patients. Generic drugs must pass a bioequivalence test to ensure they have the same therapeutic effects as the innovator counterpart and offer the same quality and safety.

However, there are some critical differences between the generics and their branded counterparts. Randomly switching between brand and generic drugs could lead to undesirable effects especially with NTIDs.

The aim of this thesis is to:

1. Investigate the use of *in-vitro* dissolution test of ciclosporin capsules to generate information about capsule rupture and drug release of brand versus generic products.
2. Develop, validate and apply a high performance liquid chromatography (HPLC) method for detecting the amount of ciclosporin in generic versus brand capsules.
3. Modify an ultra-performance liquid chromatography/mass spectrometry (UHPLC-MS) method in order that ciclosporin and its impurities could be detected simultaneously.
4. Investigate the use of *in-vitro* dissolution test of azithromycin tablets to generate information about tablet dissolution and drug release of brand versus generic products.
5. Validate a modified UHPLC-MS/MS method for quantification of azithromycin tablets.
6. Highlight any potential variations between the generic and branded innovator using an *in-vivo* bioequivalence test in man. This study will give an example of the clinical trial required to approve generic medications.

Chapter 2. Materials and final methods

2.1 Introduction

This chapter explains in detail the materials used for all experiments and the final methods used for each test.

The three main analysis systems used were a PT-DT 70 dissolution tester, HPLC and UHPLC-MS.

A dissolution test is important for quality assurance of the drug (Dressman et al., 1998). An *in-vitro* dissolution test gives the information about the capsule rupture time and drug release. The dissolved sample can be used to measure the actual mass of a drug.

HPLC is an excellent technique for measuring average drug content in tablets and capsules. It can be used for therapeutic drug monitoring (TDM) when suitable detectors such as a mass spectrometer are used. The advantages of HPLC-mass spectrometry are high sensitivity, specificity, small sample requirements, minimal sample preparation, rapid throughput, and simultaneous measurement for the drug and the possible impurities. However, HPLC with ultraviolet/fluorescent detection is also a good method to quantify and analyse the impurities in the analysis of medicines.

The UHPLC-MS method is one of the new techniques in liquid chromatography. It has the advantage of using columns with smaller particle size e.g. 1.7 µm, which result in decreasing analysis time, increasing the sensitivity, good resolution and higher efficiency (Lu et al., 2015). UHPLC-MS is required when the samples are minimal such as biopsies and blood samples (Whitman et al., 1993).

2.2 General chemicals

All general chemicals were purchased either from Sigma-Aldrich Company Ltd. (Fancy Rd., Poole, Dorset, BH12 4QH, UK) or from VWR International (Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK) unless otherwise stated.

- Ciclosporin A, lot number: BCBD2418V, catalogue number: 30024-100 mg, purity > 98.5%.
- Acetonitrile, HPLC grade, catalogue number: RH1015, batch number 12A17CA.
- Trifluoroacetic acid, catalogue number: PTS6045, batch number: PD241459.
- Azithromycin dehydrate, lot number: 020M4703V, catalogue number: PZ0007-25 mg, purity > 98%.
- Di-sodium hydrogen orthophosphate, lot number: K27313279010, product number: 10383G 500 g.
- Sodium dihydrogen orthophosphate 1-hydrate, lot number: A581121-425, product number: 102454R 500 g.
- Phosphoric acid, lot number: 59H3638, catalogue number: P-6560 500 g.
- Deionised water at purity of 18.2 MΩ.cm was obtained from ultra-water system (PURELAB® ULTRA), Windsor Court, Kingsmead Business Park, High Wycombe, HP11 1JU, UK.
- Acetonitrile, Optima™ LC/MS grade, product code: 10055454, Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, LE11 5RG, UK.
- Methanol, Optima™ LC/MS grade, product code: 10636545, Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, LE11 5RG, UK.

2.3 General equipment

- PT-DT 70 dissolution tester from PharmaTest, Hainburg, Germany (Figure 2.1).
- High quality electronic instrument: Sartorius (R 160 P) Electronic Semi-Microbalance, Sartorius Stedim, Surrey, UK.
- 20 μm Poroplast Filter Element from PharmaTest, Hainburg, Germany.
- Ultra-water system (PURELAB®ULTRA) for deionised water, ELGA, UK.

- HPLC system Jasco UV- 975 intelligent UV-VIS with Jasco AS-950 intelligent sampler injector, two Jasco PU-980 intelligent HPLC pumps and Jasco DG-2080-53 3 lines degasser.
- Jones Chromatography (Column Heater) Model 7990.
- The HPLC control data acquisition was by Chromatography Data System version 1.8.6.1 from JASCO Chrom Pass.
- HPLC used for ciclosporin separation was carried out using a reversed phase C18 column (5 µm, 125 x 4.6 mm, ACE 5) obtained from Advanced Chromatography Technologies, Aberdeen, AB25 1HF, UK.
- JENWAY spectrophotometer, Ultra-Violet/Visible, Model 6715 UV/Vis.
- UHPLC/MS used for the detection of ciclosporin impurities was carried out using a reversed phase ACQUITY UHPLC HSS T3 Column (1.8 µm, 1 mm X 150 mm), obtained from Waters Limited, 730-740 Centennial Court, Centennial Park, Elstree, Hertfordshire, WD6 3SZ, UK.
- Waters Nanoacquity UHPLC system with Waters Micromass Q-ToF Premier (Waters, UK).
- Accela UHPLC system with triple-stage-quadrupole mass spectrometry (TSQ) Vantage system (Thermo Scientific, Hemel Hempstead, UK).
- UHPLC-MS/MS azithromycin quantitation was carried out using a reversed phase Synergi HydroRP, LC Column (4 µm, 150 x 0.3 mm), obtained from Phenomenex, Queens Avenue, Hurdsfield Ind. Est., Macclesfield, Cheshire SK10 2BN, UK.

2.4 Methods used

2.4.1 HPLC experimental method for ciclosporin capsules (Test A)

Figure 2.1: PT –DT 70 dissolution tester used for dissolution test

2.4.1.1 Application of the assay

A HPLC method was applied to detect the amount of ciclosporin in generic versus brand capsules.

2.4.1.2 Method details

The extraction of active ingredients was done by a dissolution test. PT-DT 70 is the low head flip-back dissolution tester, which is (according to the manufacturer) the optimal tester for all US Pharmacopeia and European Pharmacopeia applications. It contains seven test stations with paddle stirrer adapters, fully adjustable and regulated speed from 25 to 250 rpm. Seven ciclosporin products were included in the study. Each capsule was weighed and the average weight for the capsules calculated (n = 4). Dissolution tests were done to check if all capsules met with the US Pharmacopeia 2008, (USP-31) requirements such as rupturing time within 15 min. All capsules were stored according to the conditions labelled on the package. Dissolution medium was prepared according to USP guidelines which is deionised water (500 mL). The samples were filtered using a 20 µm Filter. The HPLC experiment was carried out with a UV detection wavelength of 210 nm. The separation was carried out using a C18 column (as described in section 2.3). The column temperature was maintained at 50°C and the injection volume was 20 µL. The analytes were eluted by isocratic elution at a flow rate of 0.7 mL/min with acetonitrile and water (70:30%, v/v) and 0.03%, v/v trifluoroacetic acid, over 25 min. The data acquisition and chromatography analysis were carried out using Chromatography Data System version 1.8.6.1 from Jasco Chrom Pass. Statistical analyses and graphical presentation were carried out using Minitab-16 and Microsoft Excel 2010.

2.4.2 HPLC experimental method for ciclosporin capsules (Test B)

Same method (Test A) was applied to obtain more precise results with the following modifications:

- Eight ciclosporin products were included in this study.
- Increase the tested capsules to (n = 5).
- New calibration line.
- Peak area measurement adjustment of HPLC system, area calculated as (mV.s) instead of (mV.min)
- Sampling times increased up to 120 min and included two additional time points.

2.4.2.1 Application of the assay

A HPLC method was applied to detect the amount of ciclosporin amount in generic versus brand capsules.

2.4.2.2 Method details

Eight ciclosporin products were included in the study. Each capsule was weighed and the average weight for the capsules calculated (n = 5). Dissolution tests were done to check if all capsules met with the US Pharmacopeia 2008, (USP-31) requirements such as rupturing time within 15 min. All capsules were stored according to the conditions labelled on the package. Dissolution medium was prepared according to USP guidelines, which is deionised water (500 mL). The samples were filtered using a 20 μm Filter. The HPLC experiment was carried out with a UV detection wavelength of 210 nm. The separation was carried out using a C18 column (as described in section 2.3). The column temperature was maintained at 50°C and the injection volume was 20 μL. The analytes were eluted by isocratic elution at a flow rate of 0.7 mL/min with acetonitrile and water (70:30%, v/v) and 0.03%, v/v trifluoroacetic acid, over 25 min. The data acquisition and chromatography analysis were carried out using Chromatography Data System version 1.8.6.1 from Jasco Chrom Pass.

Statistical analyses and graphical presentation were carried out using Minitab-16 and Microsoft Excel 2010.

2.4.3 UHPLC-MS detection of impurities in ciclosporin capsules

The method used was slightly modified after Pandher et al. (2009) under the following conditions: UHPLC performed on Waters Nanoaquity UHPLC system. Separation of ciclosporin impurities was achieved on Waters Acquity UHPLC HSS T3 column (see details in section 2.3) with the following solvent system: solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile. The flow rate was 40 μL/min. The analytical run starts by 100% of solvent A for 2 min, then a gradient 100% A to 100% B over 10 min, then 100% of solvent B for 3 min, then back to 100% A over 1 min and maintain 100% of A for 4 min (Pandher et al., 2009).

Mass spectrometry conditions were as follows: mass spectrometry was performed on Waters Q-ToF Premier in positive and negative electrospray ionization modes (ESI). ESI voltages were 2.9 V in negative mode and 3.1 V in positive mode. Cone voltage was 38 V. Source temperature was 80 °C and desolvation temperature was 250 °C. Desolvation and cone gases were nitrogen with flow of 400 L/min and 30 L/min, respectively. The MS scan was adjusted to acquire between 40-1500 m/z range with scan time of 0.18 s and inter-scan delay of 0.02 s. Data were acquired in centroid mode with online lock mass correction using leucine enkephaline (MW = 556.2771) as lock mass. Instrument calibration was done by 50 mM sodium formate. A quality control ciclosporin standard was injected regularly to monitor the stability of the UHPLC system.

Data acquisition was done using Water Masslynx software (V 4.1) from Waters, UK. Data analysis of the mass detection, chromatographic peak detection, peak deconvolution, deisotoping, retention time normalisation and peak alignment were all done using MZmine software (version 2.2). Peak lists including retention, m/z and peak intensities were exported from MZmine and imported into MetaboAnalyst software (version 2.0, www.metaboanalyst.ca). Fold change, and p values were generated. Ciclosporin impurities were detected using three different online data base searches:

- PubChem Compound Database
  http://www.ncbi.nlm.nih.gov/pccompound
- KEGG Compound Database
  http://www.genome.jp/kegg/compound/
- Human Metabolome Database (HMDB)
  http://www.hmdb.ca/

2.4.4 UHPLC-MS/MS quantification for azithromycin tablets

Quantification of azithromycin was carried using a modified UHPLC-MS/MS method, modified after Rossmann et al. (2014). Azithromycin was separated on an Accela UHPLC system equipped with a Synergi HydroRP, LC column (4 µm, 150 x 0.3 mm). The column temperature was maintained at 37 °C using an Accela column oven. Gradient elution was employed using a mobile phase of 0.1% formic acid (FA) in water (A) and 0.1% FA in acetonitrile (B) as follows: A = 90% from 0-0.2 min, from 90% A to 5% over 4 min, held at 5% A for 1.4 min, from 5% to 90% A over 0.2 min, ending with 90% A for 2.7 min, all at a flow rate of 30 µL/min. (Rossmann et al., 2014) A Triple Stage Quadrupole mass spectrometry (TSQ) Vantage system equipped with an electrospray ion source was used for mass detection. Samples were analysed in Multiple Reaction Monitoring (MRM), positive mode at a spray voltage of 3500 V. Nitrogen was used as sheath at a flow rate of

20 arbitrary units. Argon was used as the collision gas with a pressure of 1.5 mTorr. The optimum transitional daughter ion mass and collision energy for azithromycin was: m/z 749.5 → 591.4 (Collision energy 20V) and internal standard roxithromycin was: m/z 837.5 → 679.4 (Collision energy 20V). Data acquisition and chromatography analysis were carried out using Xcalibur software version 2.2.

Chapter 3. Development and validation of a HPLC method for ciclosporin

3.1 Introduction

In the last few years, many questions have been raised about using generic substitutes, especially those for NTIDs. New drug safety advice announced by the Medicines & Healthcare product Regulatory Agency states that ciclosporin is such a NTID. If the patient is using one brand of ciclosporin then the same brand should be maintained for the rest of the treatment, unless change is unpreventable. This patient should be closely monitored (Medicines and Healthcare products Regulatory Agency, 2009).

Ciclosporin has significantly improved the graft survival rate in many transplantations from 60 to 80% (Opelz, 1995, Traynor et al., 2012). Ciclosporin in whole blood is routinely measured by an immunoassay supplied by some companies like Abbott (Sanghvi et al., 1989, Hamwi et al., 2000). The limit of detection for such assays is 25 ng/mL. Bioequivalence studies of ciclosporin capsules require the measurement of the drug and also the concentrations of other impurities that can lead to serious problems.

HPLC is an excellent technique for measuring average drug content in tablets and capsules. It can be used for TDM when suitable detectors such as a mass spectrometer are used. The advantages of HPLC-MS are high sensitivity, specificity, small sample requirements, minimal sample preparation, rapid throughput, and simultaneous measurement for the drug and the possible impurities. However, HPLC with ultraviolet/fluorescent detection is also a good method to quantify and analyse the impurities in the analysis of ciclosporin capsules. Like other peptides, ciclosporin can be measured by HPLC at relatively low UV absorbance wavelengths and this is considered as a gold standard for its measurement. (Burckart et al., 1990, Shaw et al., 1999, Chimalakonda et al., 2002). The HPLC-MS method is required when the samples are minimal such as biopsies and blood samples (Whitman et al., 1993). There have been various methods to measure the content of the drug in ciclosporin capsules. Some of the studies were done by cutting the capsules and dissolving them before subjecting them to an analytical HPLC system (Aziz et al., 2010). Some suggest to cut the capsule and obtain the contents and dissolve a known volume in the mobile phase before quantifying using HPLC. According to the United States Pharmacopeia, 2007, it is advised to cut about 20 capsules and extract the contents with the aid of alcohol and make up a known solution in a volumetric flask with ethanol. Then this stock is diluted to obtain a concentration of 1 mg/mL. This can then be used for the determination of ciclosporin in capsules. The other method to obtain the contents of a capsule is to aspirate using a syringe and prepare a dilution from the obtained stock. Both of these methods lack accuracy and have the possibility of producing variable results, because we cannot make sure all the contents are extracted.

A dissolution method of rupturing the capsule in the medium and then measuring the content of ciclosporin in this medium would give rise to more reproducible results.

3.2 Ciclosporin

Ciclosporin (cyclosporine A, ciclosporin A, cyclosporin A, CyA) is a lipophilic cyclic undecapeptide compound formed by a soil fungus called Tolypocladiuminflatum Gams (Golabi et al., 2003). It was discovered by Sandoz of Basel, Switzerland in the 1970s (Tribe, 1998). Ciclosporin can be prepared by synthetic or semi-synthetic ways (Hauer et al., 1994). Ciclosporin capsules contain the following inactive ingredients: Corn oil-mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-α-tocopherol USP, gelatine NF, glycerol, iron oxide black, propylene glycol USP, titanium dioxide USP, carmine, and other ingredients (Novartis Pharmaceuticals Corporation, 2011).

Chemically, ciclosporin is described as [R-[R*,R*-(E)]]-cyclic(L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl-3-hydroxy-N,4-dimethyl-L-2-amino-6-octenoyl-L-α-amino-butyryl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl) (Figure 3.1).

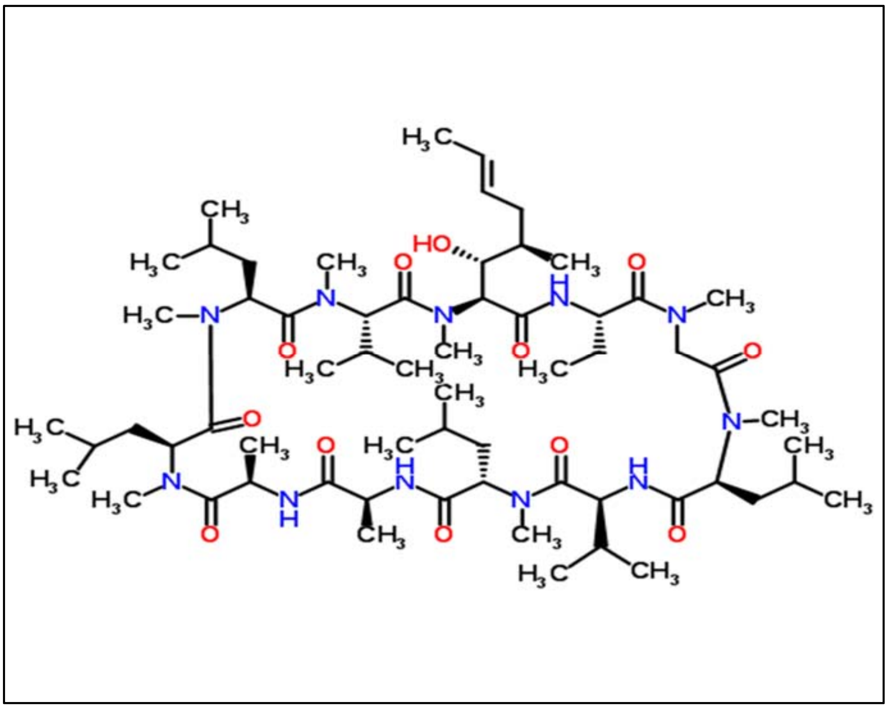

Figure 3.1: The chemical structure of ciclosporin (ChemSpider, 2015)

3.3 Applications and mode of action

Ciclosporin is an immunosuppressant agent with a narrow therapeutic index. It has been widely used since 1978 after organ or tissue transplant to prevent rejection (Allison and Eugui, 2000, Thomas et al., 2005). For auto-immune diseases such as severe rheumatoid arthritis and psoriasis, ciclosporin can also be used (Food and Drug Administration, 2011b). The donor cells used in the transplantation should be compatible with the recipient but not identical. However the response of the immune system can lead to the attack and rejection of the transplanted organ or tissue. Ciclosporin suppresses the activity of the cells of the immune system preventing such attacks or rejection of the transplanted organ or tissue (Postolache et al., 2002).

Ciclosporin binds to cyclophilin A (CycA), an intracellular protein found in the cytosol (Ryffel et al., 1991). This compound inhibits the production of lymphokines from the white blood cells via calcineurin inhibition. Lymphokines are the protein mediators responsible for T and B lymphocyte stimulation, which function against infections and foreign cells (Amor et al., 2010).

According to Johnston and Holt (2011), ciclosporin is a narrow therapeutic index drug (NTID). Irreversible kidney damage occurs at high doses. As a result, therapeutic drug monitoring is essential during ciclosporin therapy. With immunosuppressant agents, low therapeutic doses may lead to acute organ rejection. In contrast, a high dose may cause nephrotoxicity and infections. Switching among and between brands and generics (same as brand product in dosage form and route of administration, with respect to quality and safety) of these drugs can lead to undesirable effects. A study compared the biopsy-proven rate of acute rejection (BPAR) at six months after kidney transplantation between the branded immunosuppressant Neoral® and the branded-generic Gengraf™. BPAR was found to be significantly higher in patients who received Gengraf™ compared to Neoral®, 39% to 25%, respectively (Taber et al., 2005). Yet, the Food and Drug Administration considers Gengraf™ to be bioequivalent and interchangeable with Neoral® (Roza et al., 2002, Food and Drug Administration, 2009a). Another study compared the physiochemical properties of another generic immunosuppressant, tacrolimus with its counterpart brand Prograf®. It revealed that the dissolution, solubility and content uniformity profiles of generic formulations were different from that of Prograf® (Petan et al., 2008).

3.4 Pharmacokinetics and drug interactions

3.4.1 Absorption

After oral administration in man, the absorption of ciclosporin is variable. Peak plasma concentrations (C_{max}) are obtained in about 1.5-3.5 h (Novartis Pharmaceuticals Corporation, 2011). Compared to an intravenous infusion, the bioavailability of the oral dose is approximately 30-40% (Bennett and Brown, 2003).

3.4.1.1 Factors affecting ciclosporin absorption

Absorption of ciclosporin can be affected by many factors such as the physiochemical properties of the drug such as pH, dosage form and physiological factors (Burckart et al., 1986b). These physiological factors include gastric emptying rate, gastrointestinal (GI) motility, GI blood flow rate, GI pH, and first-pass metabolism. The changes in blood flow, particularly after liver transplant, may change the first-pass metabolism of the drug. Bile secretion may alter the solubilisation of ciclosporin (Postolache et al., 2002, Howland et al., 2006).

Drug-drug interactions and drug-food interactions are known to affect the GI physiological state, which can modify drug absorption. Ageing and GI disease states often lead to alterations in GI physiology and physiological reaction, resulting in further changes in the extent and rate of ciclosporin absorption. These conditions are the sources of inter- and intra-patient variability in ciclosporin absorption (Howland et al., 2006).

The oral bioavailability of ciclosporin is affected by the presence of food in the GI tract because of changes in the rate and extent of absorption. These changes occur due to gastric and bile secretion, changes in gastric motility and changes in the blood flow to the GI tract (Drewe et al., 1992). All these changes lead to alteration in the efficacy and the toxicity profile of the drug (Karalis et al., 2008).

3.4.2 Distribution

When ciclosporin enters the body's circulation, it distributes into the organs and tissues. Ciclosporin distribution is unequal due to differences in blood flow to the organs, lipid solubility, capillary permeability and accumulation at other sites. The process of distribution is reversible (Ruiz-Garcia et al., 2008). The distribution rate of the drug in the tissues is determined by perfusion. Some tissues are poorly perfused, like muscles and fat, and the distribution in such tissues is very slow especially if the tissue has a high affinity for the drug (Le, 2009). Because of the lipophilicity of ciclosporin, only a small amount is distributed in blood. The distribution within the blood is lower in lymphocytes 4-9%, and granulocytes 5-12%, and higher in plasma 33-47% and erythrocytes 41-58%. Approximately 90% of the drug undergoes plasma protein binding, mainly to lipoproteins (Novartis Pharmaceuticals Corporation, 2011).

3.4.3 Metabolism

The liver is one of the major organs responsible for drug metabolism. When the drug is absorbed into the systemic circulation it undergoes biotransformation. Most of the drug metabolism occurs in the liver, although some occurs elsewhere like the intestine. Drug metabolism is by two broad categories of enzymatic reactions, known as Phase I (oxidation, reduction, and hydrolysis) and Phase II (sulfation, glucuronidation, glutathione conjugation, acetylation, amino acid conjugation and methylation) (Neber and Roe, 2001). The products of metabolism are usually more water soluble than the original compound. The elimination of a compound means that its biological half-life is reduced and hence its potential toxicity is minimized. Metabolism has an effect on the biological activity of the drug. However, in some cases like in liver dysfunction, metabolism may increase the toxicity of the drug. Metabolism plays a central role during drug disposition as it may have a major effect on it, generally by increasing polarity and therefore water solubility facilitating excretion but it may not change the half-life ($t_{1/2}$) (period of time required for the concentration or amount of drug in the body to be reduced by one-half) (Timbrell, 2002, Verbeeck, 2008).

Ciclosporin metabolism takes place in the liver and GI tract by cytochrome CYP3A (Food and Drug Administration, 2009b), mainly by the hepatic CYP3A4 family (Howland et al., 2006, Rang et al., 2007). The metabolism includes primarily hydroxylation, demethylation, and cyclization. Thus, enzyme inducers or inhibitors of CYP3A4 alter the ciclosporin metabolism (Akhlaghi et al., 2001, Howland et al., 2006, Afshar and Nafar, 2011).

Many factors affect ciclosporin metabolism, including: species, enzyme inhibition, genetics, enzyme induction, age, dose, gender, diseases, diet, physiochemical characteristics (Craig and Stitzel, 2004, Kees et al., 2004).

3.4.4 Elimination

Once absorbed, elimination of a drug or its metabolites occurs either by liver metabolism, and/or by kidney excretion. Hepatic elimination occurs primarily by the cytochrome P450

(CYP450) family of enzymes located in the hepatic endoplasmic reticulum but may also occur by non-P450 enzyme systems, such as N-acetyl and glucuronosyl transferases. P450 enzyme systems located in gut mucosa can also significantly affect the amount of drug absorbed into the systemic circulation. Many factors alter hepatic and intestinal drug metabolism; most of these factors are usually relatively stable over time (Martindale, 1996). Most of the elimination occurs in the liver and small intestine (Burckart et al., 1986a, Novartis Pharmaceuticals Corporation, 2011) with a $t_{1/2}$ around 24 h (Rang et al., 2007). Small amounts of oral ciclosporin are eliminated by the kidney.

3.5 Uses, doses and adverse effects of ciclosporin

The FDA-labelled use of ciclosporin is for prophylaxis to prevent organ rejection in kidney, liver and heart transplantation. It is also indicated for use in severe rheumatoid arthritis and psoriasis (Food and Drug Administration, 2011b). Other uses include atopic dermatitis, severe autoimmune disease, lupus nephritis, and severe ulcerative colitis.

The initial oral dose of ciclosporin should be 4-12 h post-transplant. The total daily dose should be divided twice daily. For renal transplant patients: 9 ± 3 mg/kg/day, liver transplant patients: 8 ± 4 mg/kg/day, heart transplant patients: 7 ± 3 mg/kg/day. For rheumatoid arthritis, starting dose: 2.5 mg/kg/day and may be increased by 0.5-0.75 mg/kg/day up to maximum 4 mg/kg/day. The dose for psoriasis starts at 2.5 mg/kg/day, and may be increased by 0.5 mg/kg/day up to a maximum 4 mg/kg/day. Adverse reactions include: hypertension, hirsutism, renal dysfunction, and hypomagnesaemia (UpToDate, 2011).

3.6 Therapeutic drug monitoring of ciclosporin

Therapeutic drug monitoring (TDM) is required to enhance drug therapy in patients and it differs from one drug to another. Therapeutically monitored drugs should have a clear relationship between the drug concentration and the effect (Johnston and Holt, 1999b).

Clinical findings have shown that the therapeutic window for ciclosporin is narrow. For example, in kidney transplant patients ciclosporin therapeutic range during the first month should be from 1600 – 2000 ng/mL (Table 3.1), (Schiff et al., 2007). Therefore, monitoring of the blood concentrations of the drug and drug metabolites is considered essential in dose adjustment for high efficacy and lower toxicity (Yatscoff, 1991). In addition to having narrow therapeutic index (NTI), oral ciclosporin has a large intra- and inter-individual variability in absorption and metabolism (Holt et al., 2000, Keown, 2002).

The best way to monitor ciclosporin remains debatable (Yatscoff, 1991, Andrews and Cramb, 2002, Einollahi et al., 2011). Data suggested that single-point measurement of plasma ciclosporin after 2 h (C2) post dose can be a fast and effective method (Keown, 2002). C2 monitoring is considered to be one of the ways to measure ciclosporin concentration in kidney and liver transplant patients (Johnston and Holt, 2001, Einollahi et al., 2011, Rostami and Einollahi, 2011). The target C2 level in early treatment for kidney and liver transplant are 1500 ng/mL and 1700 ng/mL respectively (Brunet et al., 2004, Schuetz et al., 2005).

Table 3.1: Target range of ciclosporin for kidney transplant patients (Adopted from (Schiff et al., 2007)

Time	**Therapeutic range**
From 0 to 3 months	C_2 > 1700 ng/mL by day 5, 1600 – 2000 ng/mL month 1, 1400 – 1600 ng/mL month 2, 1200 – 1400 ng/mL month 3
From 3 to 12 months	C_2 from 800 – 1000 g/mL, months 4 to 6 C_2 from 600 – 800 ng/mL, months 7 to 12
More than 12 months	C_2 800 ng/mL

3.7 Experimental method development for ciclosporin analysis

3.7.1 Material and methods

3.7.1.1 General chemicals

- Please see chapter 2 (section 2.2)

3.7.1.2 General equipment

- Please see chapter 2 (section 2.3)

3.7.1.3 Collection of drug samples

All brand samples were manufactured by Novartis in Switzerland (T, S, E, J, and P) and then repackaged in the imported country. Both generics (C and I) were manufactured in India (Table 3.2).

Table 3.2: Doses and sources of ciclosporin soft gelatine capsules

Drug, dose and code	Obtained from
Ciclosporin 100 mg (S)*	Government hospital, Jeddah, Saudi Arabia
Ciclosporin 100 mg (T)*	Commercial pharmacy, Istanbul, Turkey
Ciclosporin 100 mg (P)*	Commercial pharmacy, Karachi, Pakistan
Ciclosporin 100 mg (C)	Commercial pharmacy, Colombia
Ciclosporin 100 mg (I)	Commercial pharmacy, India
Ciclosporin 50 mg (J)*	Commercial pharmacy, Amman, Jordan
Ciclosporin 50 mg (E)*	Commercial pharmacy, Cairo, Egypt

(* = branded)

3.7.2 Extraction by dissolution

A dissolution method *in-vitro* gives the information about the capsule rupture and drug release. The dissolved sample can be used to measure the actual mass of ciclosporin in branded versus generic ciclosporin capsules.

A dissolution method to analyse ciclosporin capsules was obtained from the US Pharmacopeia. Seven ciclosporin capsules were included in this study. Each capsule was weighed to check the dosage uniformity. The dissolution test was carried out under the following conditions: Temp: 37.5°C ± 0.5, 500 mL deionized water used as a medium, the paddle apparatus (Apparatus 2): 50 rpm, sampling time at (5, 10, 15, 30, 60, and 90 min), with 5 mL volume for each sample. The samples were filtered using 20 µm filters.

3.7.3 HPLC experimental method development

3.7.3.1 Identification of the Lambda max (λ_{max}) for ciclosporin measurement

A standard ciclosporin solution of 2 mg/mL was used to detect the λ_{max}. λ_{max} is the wavelength at which the maximum fraction of light is absorbed by a solution. The spectrum was compared to an acetonitrile blank, which was used as a baseline. The scan range of the spectrophotometer was set from 190 nm to 400 nm to detect maximum absorbance for ciclosporin throughout this range.

In order to determine the best absorbance wavelength for detection. Ciclosporin (2 mg/mL) in methanol was scanned using a spectrophotometer (Jenway 6715 UV/Vis). The spectrum generated showed that absorbance of ciclosporin starts at 180 nm and ends at about 250 nm (Figure 3.2). The highest absorbance was between 205 and 215 nm. Based on this result, the absorbance at 210 nm was used to detect ciclosporin in all subsequent experiments.

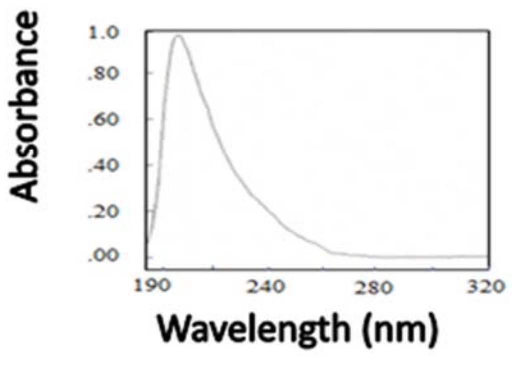

Figure 3.2: UV absorbance wavelength for 2 mg/mL ciclosporin in methanol

3.7.3.2 Selection of columns

The hydrocarbon chain forming the hydrophobic phase is usually a hydrocarbon of eighteen (C18), eight (C8) or four (C4) carbons. Peptides need longer hydrophobic chain lengths to be resolved (Carr, 2002). Various C18 columns were tested to get a good separation of ciclosporin by the HPLC method. The ACE 5 column showed the best results. All subsequent development was undertaken on this column.

3.7.3.3 Optimisation of column temperature for separation of ciclosporin

Based on literature review and the US Pharmacopeia the effect of different column temperatures on ciclosporin retention were investigated. Temperatures studied were 25 °C (Figure 3.3), 75 °C (Figure 3.4), and 50 °C (Figure 3.5). The best retention was obtained at 50 °C.

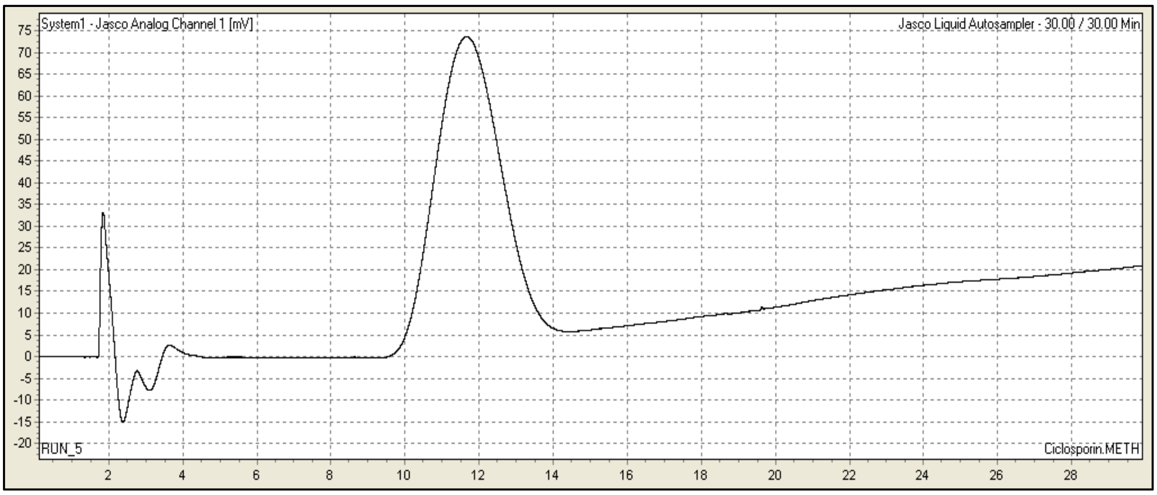

Figure 3.3: Chromatogram showing ciclosporin standard, column temperature at 25 °C

Figure 3.4: Chromatogram showing ciclosporin standard, column temperature at 75 °C

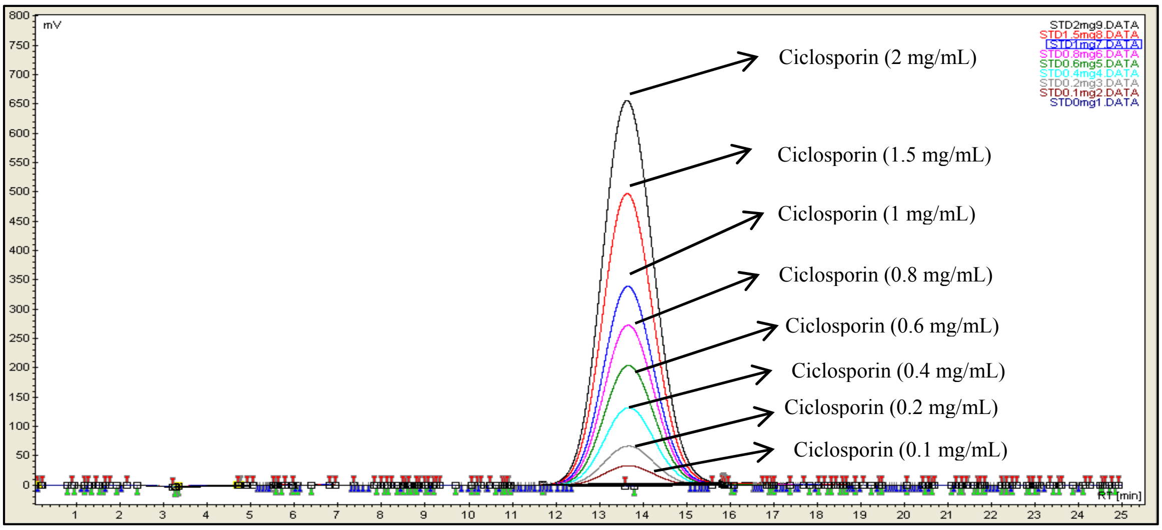

Figure 3.5: Typical chromatograms showing ciclosporin standards using the HPLC method, flow rate 0.7 mL/min, column temperature 50 °C

3.7.3.4 Optimisation of flow rate

In order to optimise the flow rate, different flow rates were studied to obtain the best resolution and peak shape, mL/min (Figure 3.6), 1.2 mL/min (Figure 3.7), and 0.7 mL/min (Figure 3.10). The best resolution was found at 0.7 mL/min.

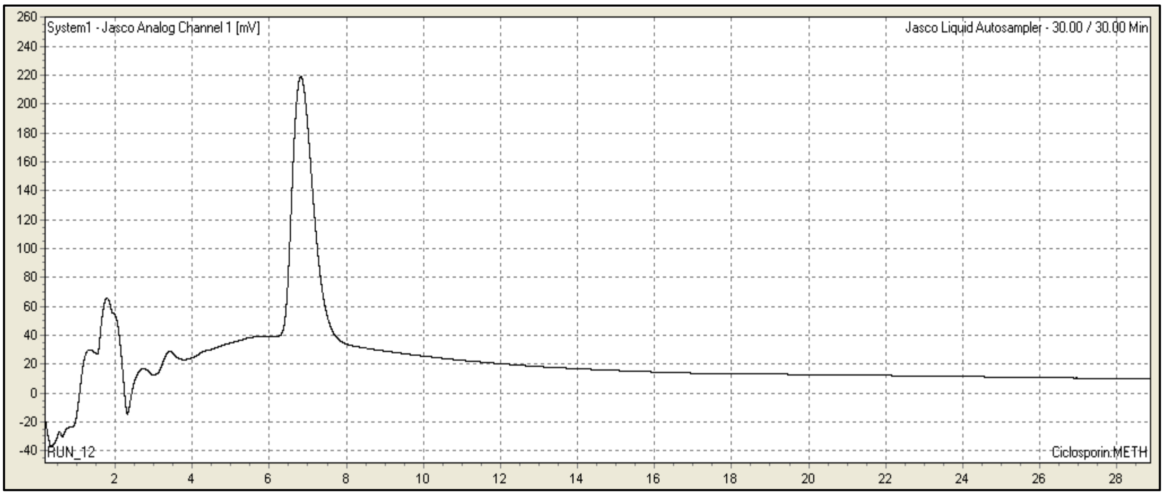

Figure 3.6: Chromatogram showing ciclosporin standard at 1 mL/min flow rate

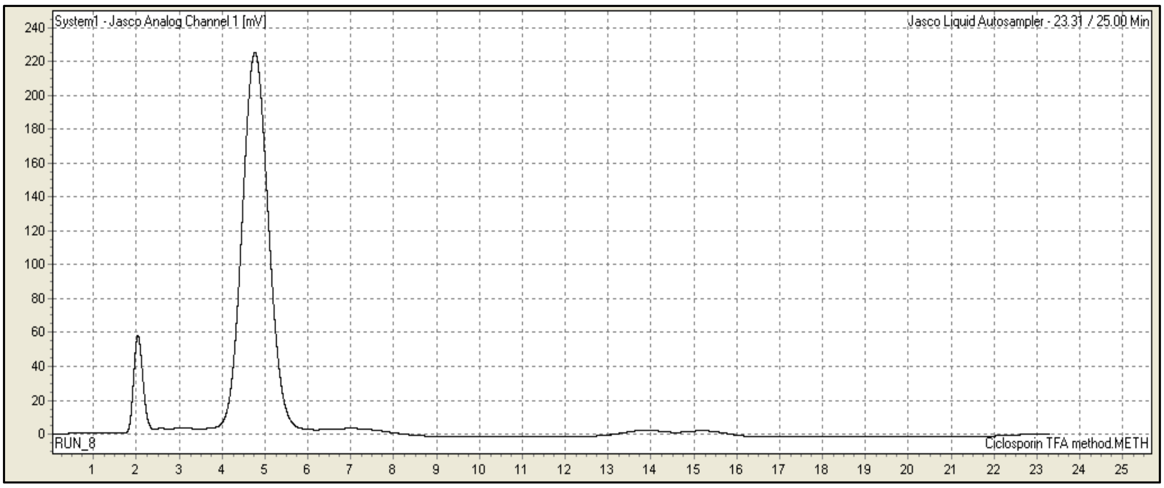

Figure 3.7: Chromatogram showing ciclosporin standard at 1.2 mL/min flow rate

3.7.3.5 Preparation of stock, calibration solutions and control samples

Ciclosporin stock solution (2 mg/mL) was prepared in the mobile phase and stored at −20ºC. The ciclosporin calibration standards were freshly prepared at the time of the experiment. Eight concentrations of ciclosporin (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5 and 2 mg/mL) were prepared in the mobile phase.

3.7.4 Calibration and linearity

Calibration standards (0.1–2 mg/mL) were injected into the HPLC system. The integrated peak areas of the ciclosporin were recorded. Linear regression of the standards was done using Microsoft Excel 2010, by plotting the peak area of ciclosporin against the ciclosporin concentration of the standards. The linearity was checked by calculating R-squared (R^2) which is a statistical measure used to calculate how close the data are to the fitted regression line values using least-square linear regression analysis.

UV detection at 210 nm was used to determine for the linearity of ciclosporin in the HPLC system. The retention of ciclosporin was achieved using the method and a typical ciclosporin chromatogram is shown in figure 3.10. A symmetrical peak shape with retention time of 13.8 min corresponded to ciclosporin retention time. The peak areas of the ciclosporin standards were 46, 92.3, 183, 275, 367, 459, 688, 916 mV.min for 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2 mg/mL respectively (Figure 3.8). The linear regression (R^2) was 1.00, regression residuals less than ±5 % (Figures 3.9).

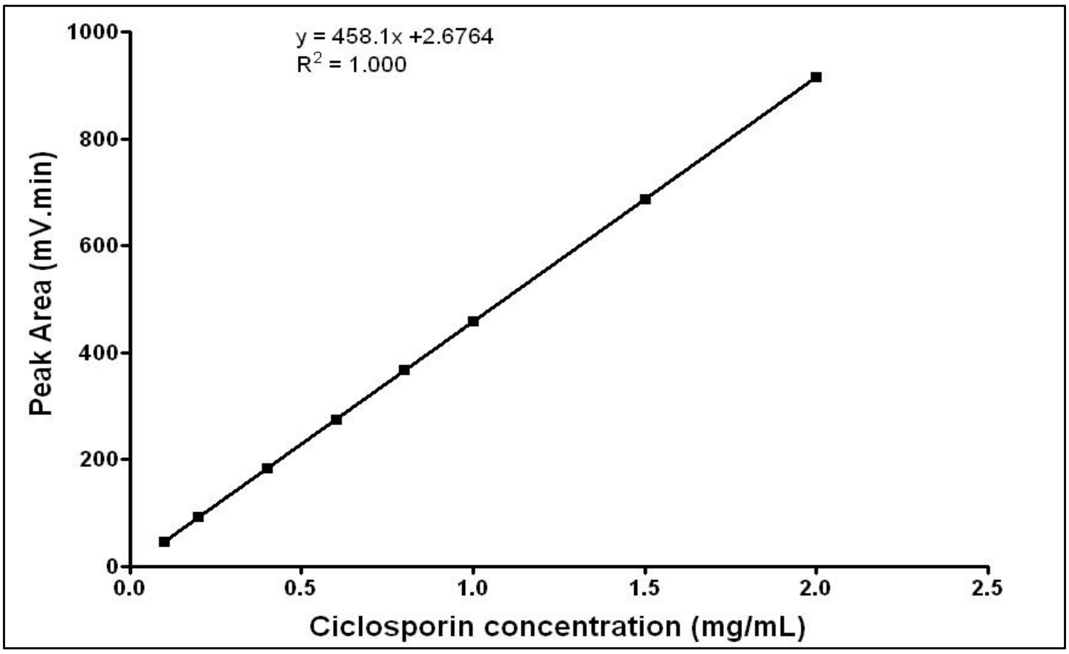

Figure 3.8: Standard curve for ciclosporin standards in mobile phase showing linear calibration line r^2 = 1 using the HPLC system (Test A)

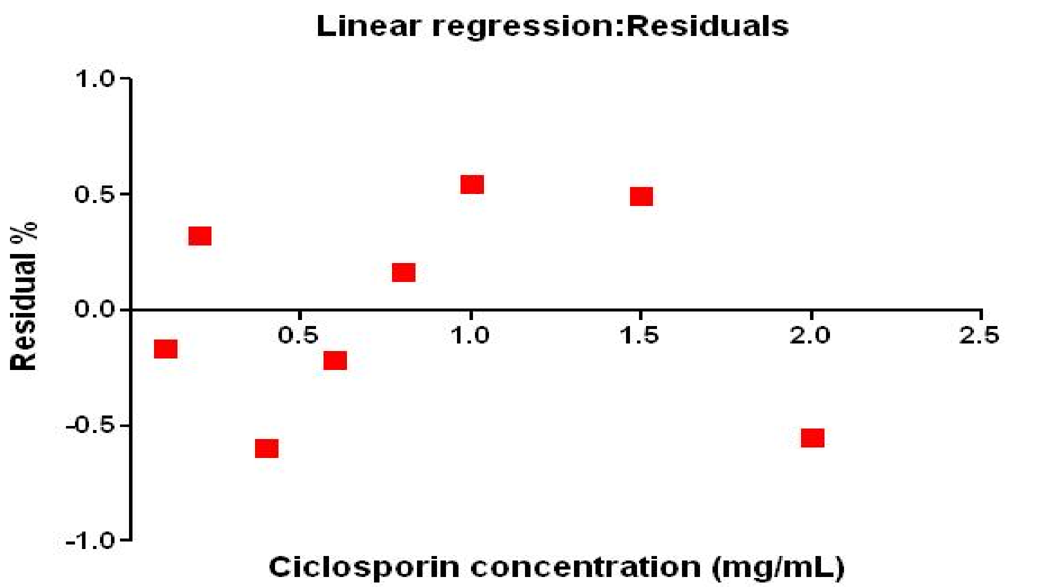

Figure 3.9: Linear regression residuals for ciclosporin standards, less than 5%

3.7.5 Method sensitivity

The detection limit of ciclosporin is the lowest concentration that can be detected using this method. The upper limit of detection is given by the point where the detector response deviates by 1% from the expected linear response.

On seven different days, the mean concentration measured of the lowest standard (0.1 mg/mL) (Figure 3.10) was 0.09 ± 0.01 mg/mL with (CV) of 11.84 %. The highest standard was 2.01 ± 0.08 mg/mL with a (CV) of 4.22 % (Table 3.3).

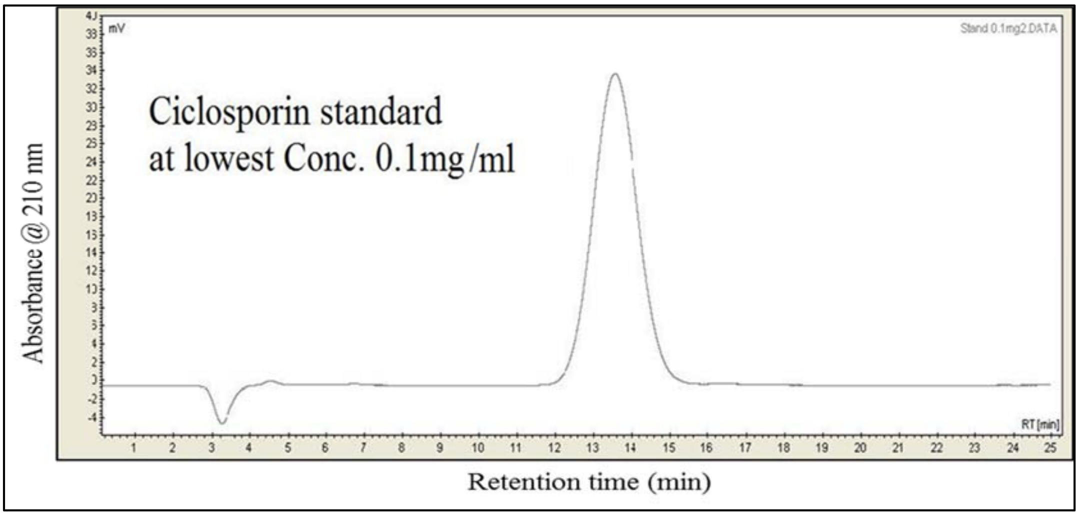

Figure 3.10: Chromatogram for the lowest ciclosporin concentration (0.1 mg/mL) at 50 °C, flow rate 0.7 mL/min

Table 3.3: Sensitivity test of ciclosporin standard showing the concentration of low and high standards on different days

Days	**Measured concentration of lowest standard (0.1 mg/mL)**	**Measured concentration of highest standard (2 mg/mL)**
1	0.07	2.21
2	0.10	1.97
3	0.10	1.99
4	0.10	2.00
5	0.10	1.99
6	0.10	2.00
7	0.10	1.97
Average	0.09	2.01
±SD	0.01	0.08

3.7.6 Method specificity

The method specificity was investigated by injecting blank mobile phase before the start of each run. The blank standards were used to control for any interfering peaks that elute at the same retention time as ciclosporin. The interfering peaks might arise from the mobile phase or the extraction process.

The method for measuring ciclosporin was specific. Blank mobile phase samples were used before, between and after each run. No significant interfering peaks and no presence of any carried over ciclosporin at the migration time of ciclosporin samples (Figure 3.11).

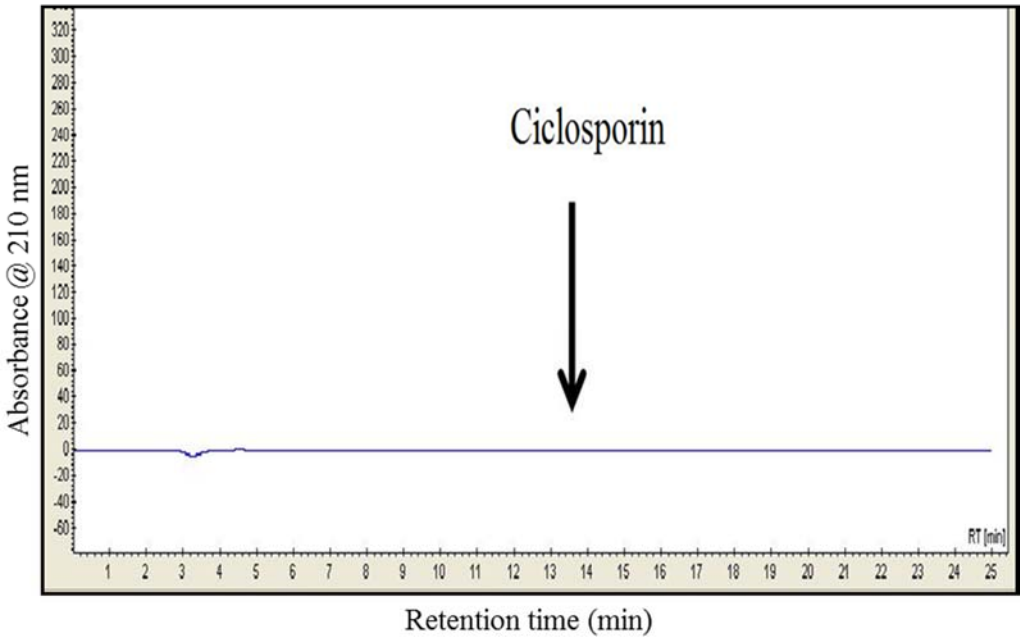

Figure 3.11: Example chromatogram to show there is no interfering peak before running ciclosporin samples. Between and after samples chromatograms also showed no interfering peaks

3.7.7 Coefficient of variation

Each ciclosporin calibration standard peak area was divided by its concentration in order to calculate the slope of the standard curve. The average and the standard deviation of the slope of all calibration standards were then calculated. Then the intra-day co-efficient of variation (CV) of this calibration line was calculated.

$$CV\% = \frac{\text{standard deviation of slope}}{\text{average of the slope}} \times 100$$

Equation 1: Calculation of coefficient of variation for ciclosporin calibration peak

3.7.8 Precision

3.7.8.1 Intra-day variability

Ciclosporin standards at 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mg were injected into the HPLC system on the same day. Table 3.4 shows the actual and measured concentrations for these samples, average, standard deviation, and the correlation of variation percentage.

Table 3.4: The mean, standard deviation (±SD) and the coefficient of variation (CV) for the intra-day variability for ciclosporin assay standard at 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mg/mL

Concentration (mg/mL)	Concentration (mg/mL)	Concentration (mg/mL)	Mean	±SD	CV% intra-day
0.1	0.09	0.10	0.10	0.002	2.22
0.2	0.20	0.21	0.20	0.007	3.64
0.4	0.39	0.40	0.40	0.009	2.30
0.6	0.61	0.57	0.59	0.024	4.05
0.8	0.81	0.82	0.82	0.004	0.51
1	1.01	1.02	1.02	0.009	0.89
1.5	1.49	1.46	1.48	0.016	1.08
2	1.98	2.00	1.99	0.010	0.51

Intra-day variability was acceptable for ciclosporin across the standard range (<4%).

3.7.8.2 Inter-day variability

Ciclosporin standards at 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mg were injected into the HPLC system. Table 3.5 shows the hypothetical and measured concentrations by HPLC analysis for these samples over six different days along with the average, standard deviation, and the coefficient of variation percentage.

Table 3.5: The mean, standard deviation (±SD) and the coefficient of variation (CV) for the inter-day variability for ciclosporin assay standards at 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mg/mL

Conc. (mg/mL)	Day1	Day2	Day3	Day4	Day5	Day6	Mean	±SD	CV%
0.1	0.10	0.09	0.10	0.09	0.10	0.10	0.10	0.001	1.41
0.2	0.20	0.20	0.21	0.20	0.21	0.20	0.20	0.005	2.45
0.4	0.38	0.39	0.40	0.39	0.40	0.38	0.39	0.013	3.18
0.6	0.62	0.61	0.57	0.61	0.57	0.62	0.60	0.020	3.35
0.8	0.82	0.81	0.82	0.81	0.82	0.82	0.82	0.003	0.33
1	1.01	1.01	1.02	1.01	1.02	1.01	1.02	0.006	0.59
1.5	1.51	1.49	1.46	1.49	1.46	1.51	1.49	0.018	1.23
2	1.97	1.98	2.00	1.98	2.00	1.97	1.98	0.014	0.68

Inter-day variability was acceptable for ciclosporin across the standard range (< 5%).

3.7.9 Inaccuracy

The inaccuracy of the assay was determined by measuring the difference between actual (hypothetical) and measured concentration of each ciclosporin calibration standard. The concentration difference was then divided by the actual concentration and multiplied by 100 (Equation 2).

$$\text{Inaccuracy} = \frac{\text{Actual concentrat ion - measured concentrat ion}}{\text{Actual concentrat ion}} \times 100$$

Equation 2: Inaccuracy percentage calculations of hypothetical and measured ciclosporin concentrations

The inaccuracy of the method was measured by calculating the difference between actual and measured concentration of each ciclosporin calibration standard (Table 3.6).

Table 3.6: Inaccuracy of the assay was < 4% of the true value at standard curve concentrations range from 0.1 to 2 mg/mL of ciclosporin

Conc. (mg/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Average**	**±SD**
0.1	-32.69	0.19	2.98	0.19	2.98	1.70	-4.10	14
0.2	-29.27	0.12	5.41	0.12	5.41	1.15	-2.83	13
0.4	-23.97	0.90	2.38	0.90	2.38	4.66	-2.12	11
0.6	11.65	1.93	3.75	1.93	3.75	3.38	4.40	4
0.8	2.24	2.26	3.01	2.26	3.01	2.52	2.55	0
1	-1.96	1.64	2.94	1.64	2.94	1.96	1.52	2
1.5	-18.14	0.53	2.05	0.53	2.05	0.69	-2.04	8
2	10.62	0.61	0.11	0.61	0.11	1.41	2.24	4

On the first day the inaccuracy of the 3 lowest concentrations was above the acceptable level due to an unknown reason. However in the subsequent 6 experiments the inaccuracies were within the acceptable range (less than 15%).

3.7.10 HPLC system carryover

The system carryover was checked by running blank samples between actual samples. The blank sample was checked for the presence of any carried over ciclosporin. Calibration standards were also randomised and the calibration curve linearity was then checked in order to detect any carryover.

3.7.11 Statistical analysis and graphic presentation

Graphic presentations and the statistical analyses were done using Excel 2010 for Windows version, 14.0.6112.500 (32-bit) and Minitab version, 16.

3.7.12 Dissolution extraction recovery

The recovery was calculated by dividing the actual volume over the total volume and then multiplied by the concentration (Equation 3).

$$\text{Recovery} = \frac{\text{Actual volume}}{\text{Total volume}} \times \text{Concentrat ion}$$

Equation 3: Recovery calculation of ciclosporin standards

3.7.13 Stability

Freeze thaw stability was done for three ciclosporin calibration standards stored at −20°C, thawed and re-frozen weekly for three weeks.

This test was to check the stability of ciclosporin standards in stock solutions. Ciclosporin standards were stable for the whole period of the experiment. Two different concentrations of 0.4 and 0.6 mg from day 2, 4, and 6 were measured (Table 3.7).

Table 3.7: The average concentration (±SD) and coefficient of variation (CV) of ciclosporin stability in stock solution for day 2, 4 and 6

Conc. (mg/mL)	Day 2	Day 4	Day 6	Average	±SD	CV%
0.4	0.39	0.39	0.38	0.39	0.009	2.22
0.6	0.61	0.61	0.62	0.61	0.005	0.82

Table 3.8: The inaccuracy of ciclosporin stability in stock solution for day 2, 4 and 6

Inaccuracy (mg/mL)	Day 2	Day 4	Day 6	Average	±SD
0.4	0.90	0.90	4.66	2.15	2.17
0.6	1.93	1.93	3.38	2.41	0.84

The ciclosporin standards were stable for freeze-thaw cycles. Average measured concentration for 3 cycles of 0.4 and 0.6 mg of ciclosporin was 0.39 and 0.61 respectively (Table 3.7). The CV% of 0.4 and 0.6 mg of ciclosporin standards were less than 3%. Inaccuracy of measured concentration was less than ± 3% (Table 3.8).

Chapter 4. Application of the HPLC method to the measurement of ciclosporin from different countries

4.1 Overall description of the method

This study describes the development, validation and application of a simple HPLC method for the determination of ciclosporin. Seven ciclosporin products were obtained from commercial pharmacies and hospitals for inclusion in this study.

Ciclosporin capsules were dissolved in dissolution tester. Samples were obtained at different time intervals. The contents of the dissolved sample were separated by injecting 20 μL of the samples into the HPLC system. The column was held at 50 ± 0.3°C. The λ_{max} for ciclosporin at 210 nm.

4.2 Results of HPLC method for ciclosporin capsules (Test A)

4.2.1 Dissolution test for ciclosporin

The capsule rupture was determined by visual observation of the capsule shell and/or by the release of capsule content. Rupturing time was determined for each capsule by using a stopwatch. The results from the dissolution test showed that the average rupture times for ciclosporin capsules of generic C, brand T, brand J, brand E, generic I, brand P and brand S were (mean ± SD) 1.56 ± 0.02, 5.07 ± 0.02, 5.11 ± 0.01, 5.29 ± 0.02, 5.34 ± 0.03, 5.37 ± 0.02 and 5.43 ± 0.02 min, respectively, (n = 4). A sample of medium was tested using HPLC before putting the capsule into the vessel to insure no ciclosporin contamination of the medium. The chromatogram shows that there was no ciclosporin peak (Figure 4.1) and confirms the absence of ciclosporin in the medium. In this study all the capsules met the USP requirements, rupturing within 15 min (Table 4.1).

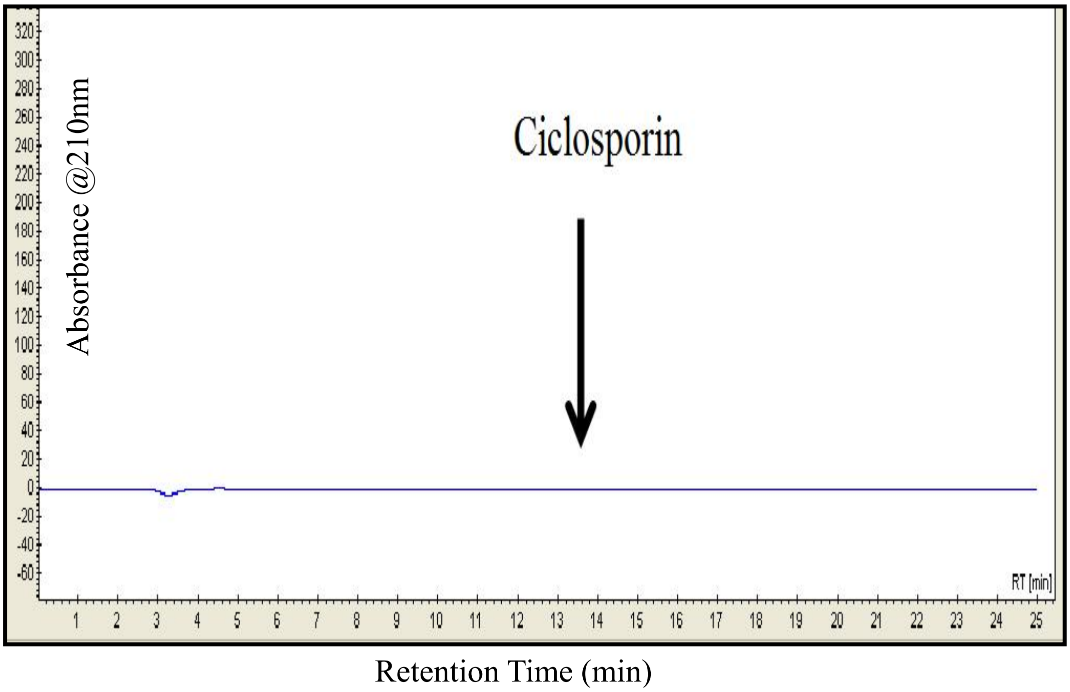

Figure 4.1: Chromatogram of medium sample showing no ciclosporin contamination of the dissolution tester

Table 4.1: Average rupture time for ciclosporin capsules (n = 4)

Ciclosporin capsule	**Average rupture time (min)** ± SD
Generic C	1.56 ± 0.02
Brand T	5.07 ± 0.02
Brand J	5.11 ± 0.01
Brand E	5.29 ± 0.02
Generic I	5.34 ± 0.03
Brand P	5.37 ± 0.02
Brand S	5.43 ± 0.02

Figure 4.2, shows a typical chromatogram of ciclosporin concentrations in 5 different capsules (Brands S, T, and P, Generics I, and C) which were detected at the same retention time. Although, standard ciclosporin gives one peak, while in some tested capsules more peaks can be seen.

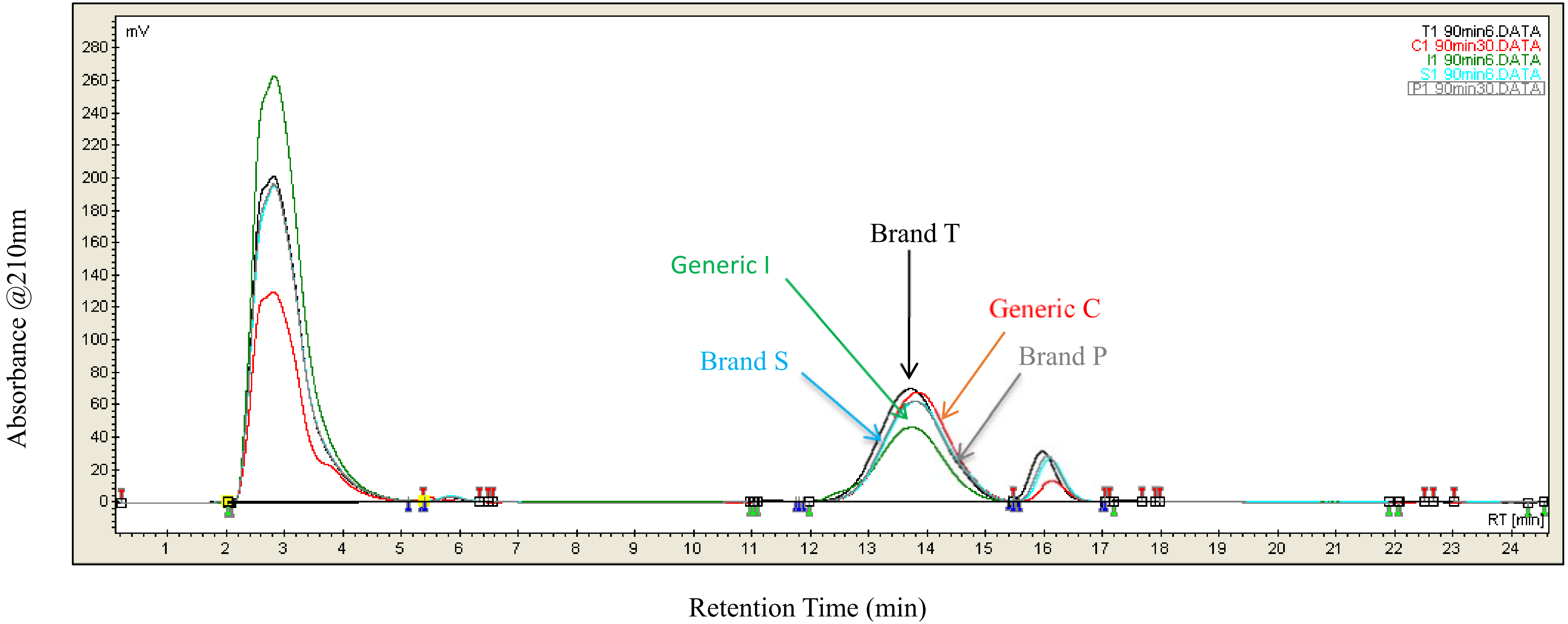

Figure 4.2: A typical overlaid chromatogram showing the varying ciclosporin concentration in 5 different capsules (Brands S, T, and P, Generics I, and C)

4.2.2 Drug recovery (from capsules)

This was done in order to confirm that the dissolution test successfully recovered all the capsule content after 60 and 90 min.

Maximal recovery (99 ± 0.4%) was after 60 min. Recovery after 90 min was 100 ± 0.5% (Table 4.2).

Table 4.2: The average recovery percentage of ciclosporin mass amount after 60 and 90 min of the dissolution test (n = 4)

Recovery Average		
Ciclosporin	**60 min**	**90 min**
Brand T	99 ± 0.04 %	100 ± 0.05 %
Brand P	91 ± 0.03 %	90 ± 0.03 %
Brand S	88 ± 0.04 %	90 ± 0.07 %
Generic C	86 ± 0.05 %	91 ± 0.01 %
Brand J	84 ± 0.02 %	83 ± 0.05 %
Brand E	76 ± 0.05 %	81 ± 0.04 %
Generic I	69 ± 0.04 %	69 ± 0.08%

4.2.3 Final results of Test A

This method was successfully applied to measure the actual concentration in each ciclosporin product using HPLC analysis. Sampling time was done on six different intervals in order to check for ciclosporin release from capsules. All brands (T, S, E, J, P) and one generic (C) showed more than 80% of labelled amount in ciclosporin capsules after 90 min of the dissolution test giving 100 ± 0.05, 90 ± 0.07, 81 ± 0.04, 83 ± 0.05, 90 ± 0.03, and 91 ± 0.01 % (±SD), respectively. One generic (I), showed less than the minimum percentage of labelled amount 69 ± 0.08% (Figure 4.3). Relative to the brand (T), statistical analysis showed significant differences ($p<0.0001$) of the mean percentage content between brand and generic, the 95% confidence interval (CI) range for the brands (S, E, J, P) were (80.1-101.8), (72.2-91.8), (73.4-93.3), (80.2-101.9), respectively, and (80.3-102.1), (61.3-77.9), for the generic (C) and (I), respectively (Table 4.4).

Table 4.3: Average percentage content of ciclosporin in capsules (Test A)

Drug Name	Country of Origin	Average weight (g), n = 4	Sampling time (min) and Average content %, n = 4					
			5 min	10 min	15min	30 min	60 min	90 min
Neoral®*	Turkey	1.50	6.7%	55.8%	98.3%	98.6%	98.8%	100.0%
Neoral®*	Saudi	1.51	0.4%	57.8%	85.0%	91.9%	88.4%	90.4%
Neoral®	Jordan	1.67	3.2%	65.3%	77.6%	80.5%	84.2%	82.8%
Neoral®	Egypt	1.69	3.1%	13.5%	55.7%	74.8%	76.2%	81.4%
Neoral®*	Pakistan	1.50	6.9%	79.2%	93.3%	89.3%	91.3%	90.4%
Generic C*	Colombia	1.32	85.0%	94.7%	88.0%	89.5%	86.1%	90.5%
Generic I*	India	1.60	4.6%	47.8%	72.0%	65.7%	68.9%	69.3%

*Ciclosporin 100 mg capsules. Jordan and Egypt capsules contain 50 mg

Table 4.3 shows the average percentage of ciclosporin mass amount at different time intervals, reference capsule (Turkey) showed 100% mass amount after 90 min, (n = 4)

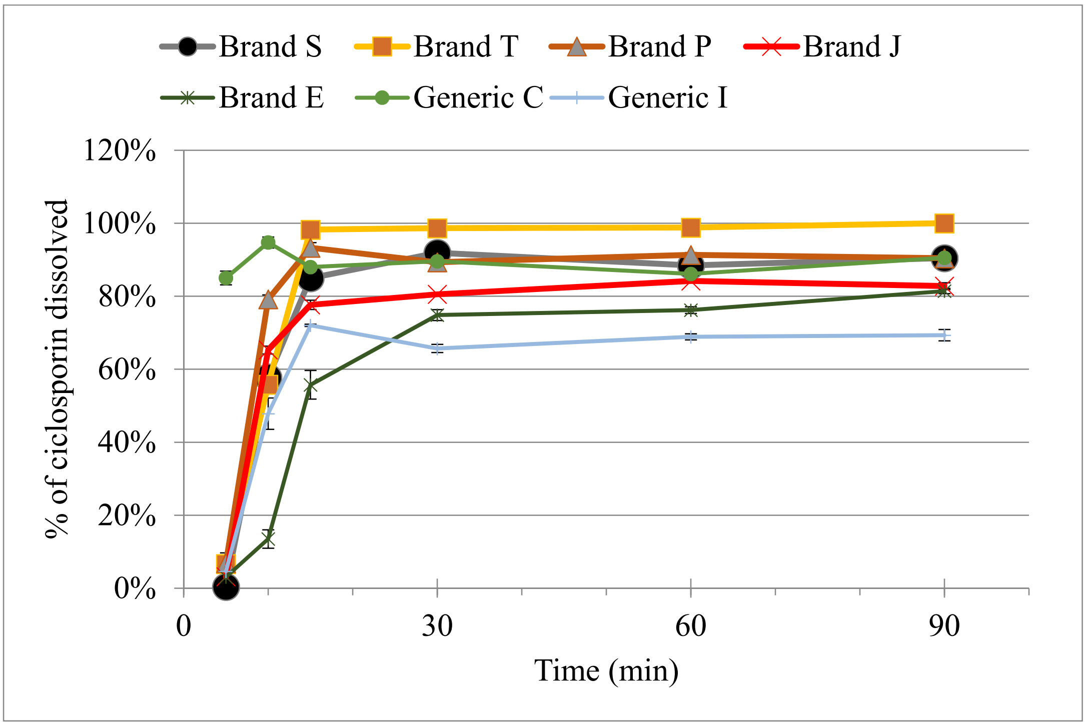

Figure 4.3: Percentage of dissolved ciclosporin in brands and generics

Table 4.4: The average percentage of ciclosporin mass amount, standard deviation, coefficient of variation and 95% CI based on reference capsule (T) 100% mass amount, (n = 4)

Ciclosporin capsule	**N**	**% of mass amount at 90 min (mg/mL)**	**± SD**	**CV (%)**	**95% confidence interval**
Generic I (100 mg)	4	69.3	7.6	11	(61.3-77.9)
Brand E (50 mg)	4	81.4	3.6	4	(72.2- 91.8)
Brand J (50 mg)	4	82.8	4.7	6	(73.4-93.3)
Brand S (100 mg)	4	90.4	6.9	8	(80.1-101.8)
Brand P (100 mg)	4	90.4	3.3	4	(80.2-101.9)
Generic C (100 mg)	4	90.5	1.2	1	(80.3-102.1)

Table 4.4 shows that ciclosporin has variable mass labelled amount compared to the reference capsule. Even with brand capsules, ciclosporin showed variability. One generic failed to meet the minimum requirement, indicating availability of substandard and/or counterfeit ciclosporin in the market.

4.3 Dissolution test for ciclosporin Test B

Same method (Test A) was applied to obtain more precise results with the following modifications: Eight ciclosporin products were included in this study, tested capsules to (n = 5), peak area measurement adjustment of HPLC system, area calculated as (mV.s) instead of (mV.min), and the sampling times increased up to 120 min to included two additional points.

The results from the dissolution test showed that the rupture times for ciclosporin capsules of generic (Col), brand (Egy), generic (Ir), brand (Jor), generic (M), brand (Pak), brand (Sa) and brand (TK), showed that they all met the USP requirements, rupturing within 15 min.

4.3.1 Standard curve and linearity

Figure 4.4 shows a typical standard curve for ciclosporin. Coefficient of correlation ranged between 0.997 and 1. The mean slope was 55.5 ± 0.4 mV/mg for ciclosporin (n = 8).

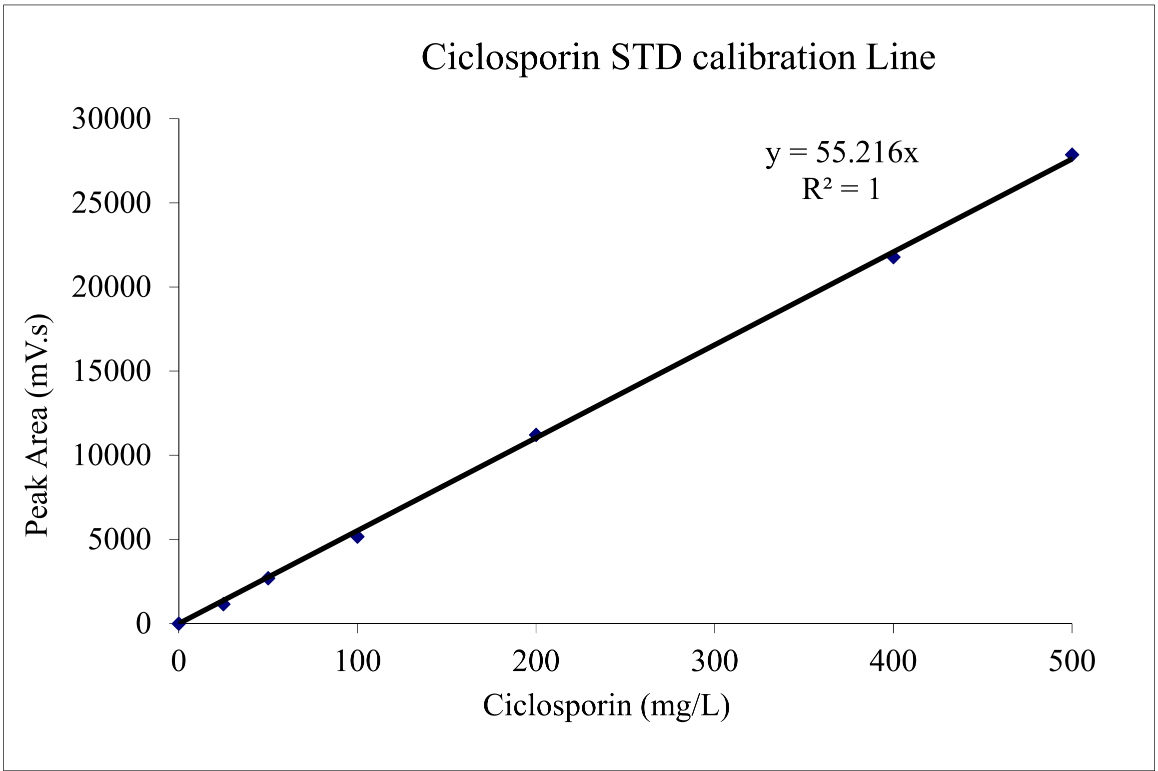

Figure 4.4: Standard curve for ciclosporin standards in mobile phase showing linear calibration line $r^2 = 1$ (Test B)

4.3.2 Sensitivity

On eight different days, the mean concentration measured of the lower standard (25 mg/L) was 22.3 ± 0.97 mg/L with a CV of 4.4%., and for the highest standard (500 mg/L) it was 500.4 ± 2.6 mg/L with a CV of 0.5% (Table 4.5).

Table 4.5: Sensitivity test of ciclosporin standard showing the concentration of low and high standards on different days (Test B)

Days	Measured concentration of lowest standard (25 mg/L)	Measured concentration of highest standard (500 mg/L)
1	23.8	496.8
2	21.6	503.0
3	23.0	498.1
4	21.4	501.2
5	23.3	499.2
6	22.3	499.2
7	22.0	501.3
8	21.0	504.6
Average	22.3	500.4
±SD	1.0	2.6

4.3.3 Specificity

The method was specific. There were no significant interfering peaks before, between and after each run.

4.3.4 Precision

4.3.4.1 Intra-day variability

Ciclosporin standards at 25, 50, 100, 200, 400, and 500 mg/L were injected into the HPLC system on the same day. Table 4.6 shows the actual and measured concentration for these samples, average, standard deviation, and the correlation of variation percentage.

Table 4.6: The average, standard deviation (±SD) and the coefficient of variation (CV) for the intra-day variability for ciclosporin assay standard at 25, 50, 100, 200, 400, and 500 mg/L

Concentration (mg/L)	Concentration (mg/L)	Concentration (mg/L)	Mean	±SD	CV% intra-day
25	21.4	21.0	21.2	0.3	1.4
50	49.3	48.9	49.1	0.3	0.6
100	93.5	93.8	93.6	0.2	0.2
200	202.6	203.2	202.9	0.4	0.2
400	399.1	394.6	396.9	3.2	0.8
500	501.2	504.6	502.9	2.4	0.5

Intra-day variability was acceptable for ciclosporin across the standard range (<2%)

4.3.4.2 Inter-day variability

Ciclosporin standards at 25, 50, 100, 200, 400 and 500 mg/L were injected into the HPLC system. Table 4.7 shows the hypothetical and measured concentration by HPLC analysis for these samples over eight different days along with the average, standard deviation, and the coefficient of variation percentage.

Table 4.7: The average, standard deviation (±SD) and the coefficient of variation (CV) for the inter-day variability for ciclosporin assay standards at 25, 50, 100, 200, 400, and 500 mg/L

Conc. (mg/L)	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Mean	±SD	CV%
25	23.8	21.6	23.0	21.4	23.3	22.3	22.0	21.0	22.3	1.0	4.4
50	52.4	49.2	50.9	49.3	49.7	49.9	49.8	48.9	50.0	1.2	2.3
100	119.9	120.6	119.6	93.5	119.4	118.2	119.1	93.8	113.0	12.0	10.6
200	198.7	199.0	198.1	202.6	198.3	199.7	200.8	203.2	200.0	1.9	1.0
400	399.5	391.9	398.4	399.1	397.1	396.8	393.4	394.6	396.3	2.8	0.7
500	496.8	503.0	498.1	501.2	499.2	499.2	501.3	504.6	500.4	2.6	0.5

Inter-day variability was acceptable for ciclosporin across the standard range (<11%)

4.3.5 Inaccuracy

The inaccuracy of the method was measured by calculating the difference between the actual and measured concentration of each ciclosporin calibration standard (Table 4.8)

Table 4.8: Inaccuracy of the assay was < 13% of the true value at standard curve concentrations ranging from 25 to 500 mg/L of ciclosporin

Conc. (mg/L)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Average	±SD
25	-4.94	-13.65	-8.05	-14.32	-6.91	-10.67	-12.18	-16.00	-10.84	4
50	4.87	-1.64	1.86	-1.39	-0.59	-0.24	-0.46	-2.22	0.02	2
100	19.95	20.60	19.58	-6.54	19.40	18.22	19.14	-6.21	13.02	12
200	-0.67	-0.48	-0.94	1.30	-0.83	-0.16	0.39	1.59	0.02	1
400	-0.13	-2.02	-0.41	-0.21	-0.73	-0.81	-1.66	-1.36	-0.92	1
500	-0.64	0.59	-0.37	0.24	-0.15	-0.16	0.27	0.93	0.09	1

4.3.6 Drug recovery from capsules

This was done in order to confirm that the dissolution test successfully recovered all the capsule content after 90 and 120 min. Maximal recovery (100 ± 0.03%) was after 90 min. Recovery after 120 min was 100 ± 0.01% (Table 4.9).

Table 4.9: The average recovery percentage of ciclosporin mass amount after 90 and 120 min of the dissolution test (n = 5)

Recovery Average		
Ciclosporin	**90 min**	**120 min**
Brand TK	100 ± 0.03 %	100 ± 0.01 %
Brand Pak	94 ± 0.08 %	93 ± 0.09 %
Brand Sa	99 ± 0.02 %	99 ± 0.01 %
Generic Col	85 ± 0.02 %	83 ± 0.01 %
Brand Jor	84 ± 0.09 %	84 ± 0.10 %
Brand Egy	92 ± 0.01 %	91 ± 0.01 %
Generic Ir	97 ± 0.02 %	95 ± 0.02%
Generic M	54 ± 0.10 %	56 ± 0.08%

4.3.7 Stability

This test was done to check the stability of ciclosporin standards in stock solutions. Ciclosporin standards were stable for the whole period of the experiment. Two different concentrations of 50 and 200 mg/L from day 2, 4, and 6 were measured (Table 4.10).

The ciclosporin standards were stable for freeze-thaw cycles. The average measured concentration for 3 cycles of 50 and 200 mg/L of ciclosporin was 49.45 and 200.44 mg/mL, respectively. The CV% of 50 and 200 mg/L ciclosporin standards was less than 1%. Inaccuracy of the measured concentration was less than ±2%.

Table 4.10: The average concentration (±SD) and coefficient of variation (CV) ciclosporin stability in stock solution for day 2, 4, and 6

Conc. (mg/L)	Day 2	Day 4	Day 6	Average	±SD	CV%
50	49.18	49.30	49.88	49.45	0.37	0.75
200	199.04	202.60	199.69	200.44	1.90	0.95

4.3.8 Final results of Test B

The same method was successfully applied to measure the actual concentration in each ciclosporin product using HPLC analysis. Sampling time was done on nine different intervals in order to check for ciclosporin release from capsules. All brands (TK, Sa, Egy, Jor, Pak) and two generics (Col, Ir) showed more than 80% of labelled amount in ciclosporin capsules after 90 min of the dissolution test giving 100 ± 0.03, 99 ± 0.02, 92 ± 0.01, 84 ± 0.09, 94 ± 0.08, 85 ± 0.02 % and 97 ± 0.02 (±SD), respectively. One generic (M), showed less than the minimum percentage of labelled amount 54 ± 0.10% (Figure 4.5). Table 4.11 shows the average percentage of ciclosporin mass amount at different time intervals, reference capsule (Turkey) showed 100% mass amount after 90 min, (n = 5). Relative to the brand (TK), statistical analysis showed significant differences ($p<0.0001$) of the mean percentage content between brand and generic, the 95% CI range for the brands (Sa, Egy, Jor, Pak) were (97-102), (90-93), (72-95), and (85-103), respectively, and (83-87), (94-99), (41-67) for the generics (Col, Ir, M) respectively (Table 4.12).

Table 4.11: Average percentage content of ciclosporin in capsules (Test B)

Drug name	Country of origin	Average weight (g), n = 5	Sampling time (min) and average content %, n = 5								
			5	10	15	20	30	45	60	90	120
Neoral®*	Turkey	1.50	4 %	60 %	81 %	93 %	97 %	98 %	99 %	100 %	100 %
Neoral®*	Saudi	1.50	2 %	53 %	71 %	88 %	92 %	96 %	97 %	99 %	99 %
Neoral®	Jordan	1.68	6 %	68 %	79 %	80 %	80 %	79 %	83 %	84 %	84 %
Neoral®	Egypt	1.68	6 %	14 %	67 %	80 %	84 %	88 %	89 %	92 %	91 %
Neoral®*	Pakistan	1.49	2 %	72 %	86 %	89 %	93 %	95 %	95 %	94 %	93 %
Generic Col*	Colombia	1.32	81 %	87 %	88 %	87 %	88 %	87 %	87 %	85 %	83 %
Generic Ir*	Iran	1.55	3 %	44 %	72 %	83 %	93 %	98 %	99 %	97 %	95 %
Generic M*	Morocco	1.61	0 %	33 %	51 %	55 %	55 %	57 %	58 %	54 %	56 %

*Ciclosporin 100 mg capsules. Jordan and Egypt capsules contain 50 mg

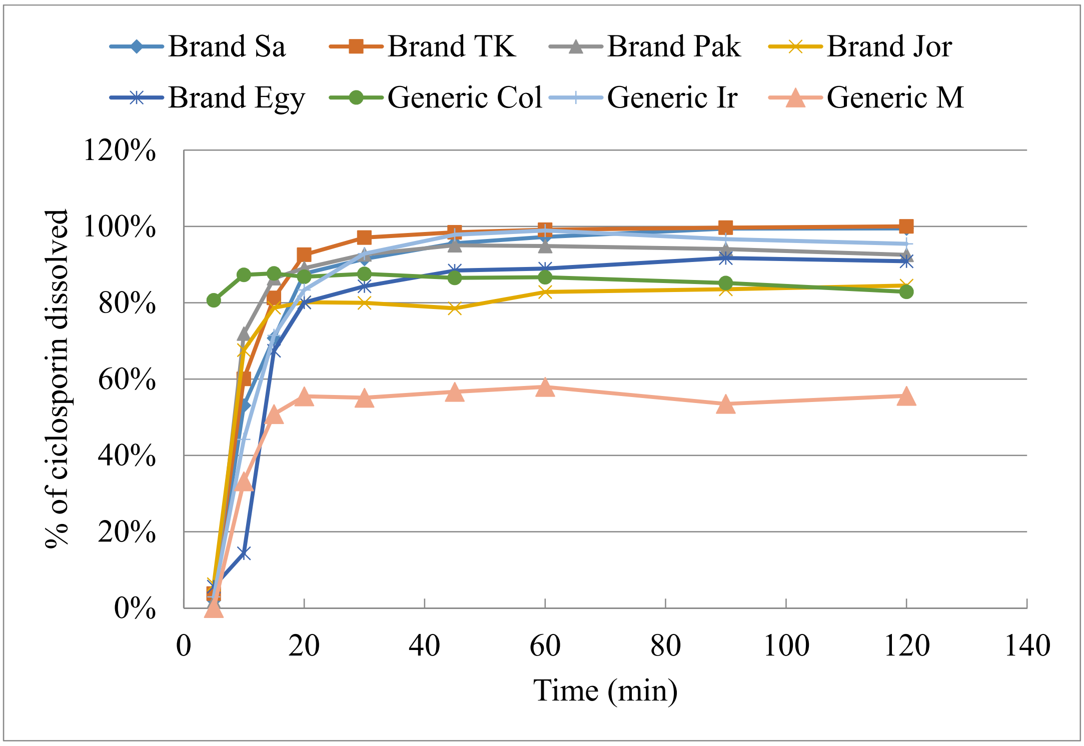

Figure 4.5: Percentage dissolved ciclosporin in brand and generics (n = 5)

Table 4.12: The average percentage of ciclosporin mass amount, standard deviation, coefficient of variation and 95% CI based on reference capsule (TK) 100% mass amount, (n = 5)

Drug	**N**	**% of mass amount at 90 min (mg/L)**	**± SD**	**CV (%)**	**95% Confidence Interval**
Brand Egy	5	92	1.3	1	(90- 93)
Brand Jor	5	84	9.1	11	(72-95)
Brand Pak	5	94	7.5	8	(85-103)
Brand Sa	5	99	1.9	2	(97-102)
Generic Col	5	85	1.9	2	(83-87)
Generic Ir	5	97	2.1	2	(94-99)
Generic M	5	54	10.0	20	(41-67)

4.4 UHPLC-MS detection of impurities in ciclosporin capsules generic (Col)

The chemical contents of ciclosporin capsules (Generic Col) were studied using an untargeted metabolomic UHPLC-MS-based approach. Then, all the chemical contents of generic (Col) capsules were compared to a reference capsule, brand (TK), (reference product). A representative TIC is shown in figure 4.6 for the reference compund TK. In particular, one impurity was detected at 8 min with high ciclosporin/impurity peak ratio suggesting a clean product. In contrast, figure 4.7 shows poor prodcut with multiple impurities at 2, 3, 4, 9 and 11 min with very small ciclospotin/impurities peak ratios indicating a poor quality product. In addition, one of the impurities at 9.8 min had a putative identity of N-(8-Aminooctyl)-N5-(diaminomethylene)-L-ornithinamide, an amino acid which could be related to ciclosporin polypeptide.

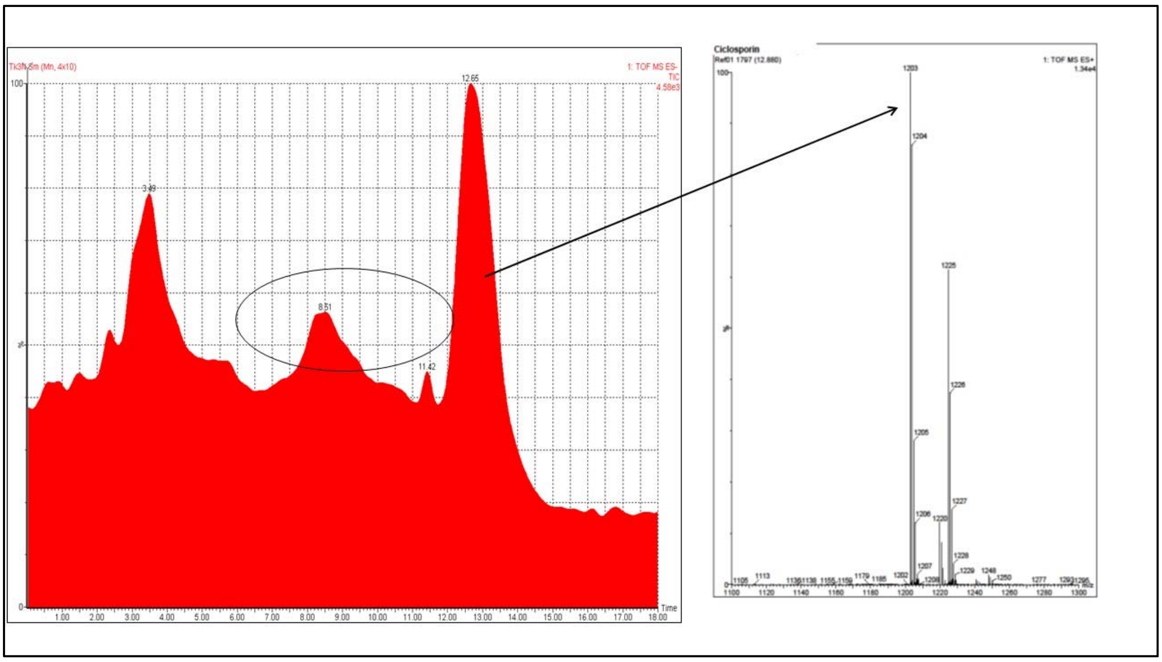

Figure 4.6: The LC-MS total ion chromatogram (TIC) of an example of a relatively clean product with a clear ciclosporin peak at 12 min and small impurity at 8 min

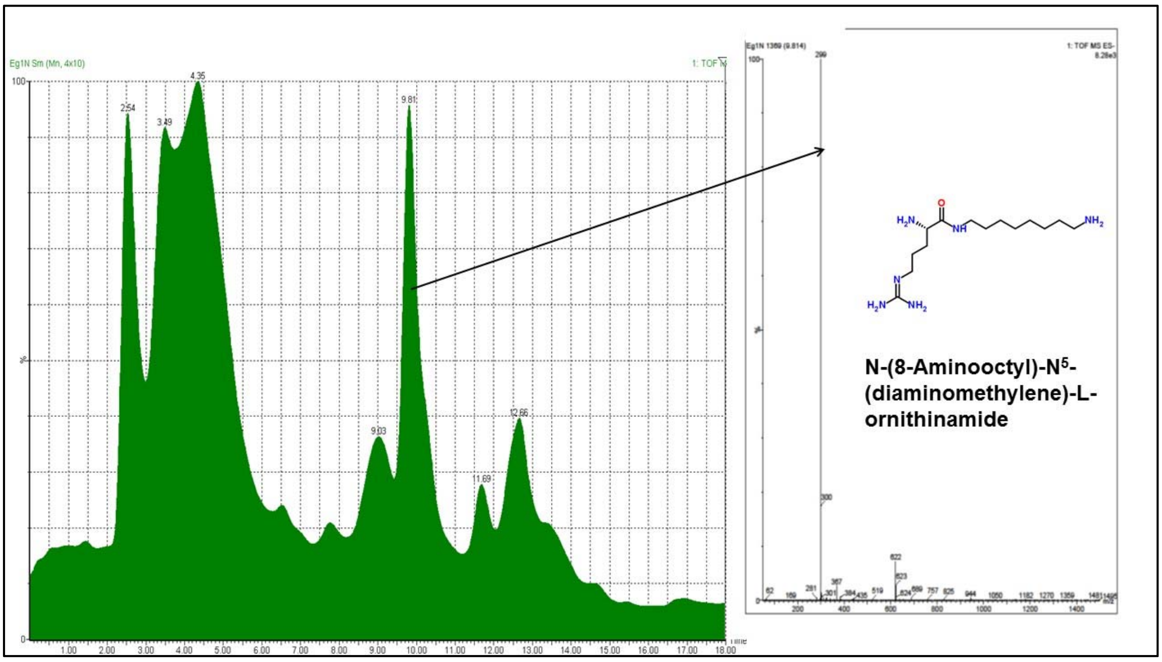

Figure 4.7: The LC-MS TIC of an example of a poor product with small peak of ciclosporin at 12 min and high impurity at 9 min

Figure 4.8 shows clear separation between the samples derived from the generic product (generic Col) compared to the reference product (TK) using principal component analysis (PCA). PCA is an unsupervised multivariate analysis aimed at reducing high-dimensional data into fewer dimensions called principal components (PCs). Each dimension is called a PC, which represents a linear combination of original variables. The first PC is the component that accounts for the highest variability in the data. Then, each subsequent PC accounts for the remaining data variability.

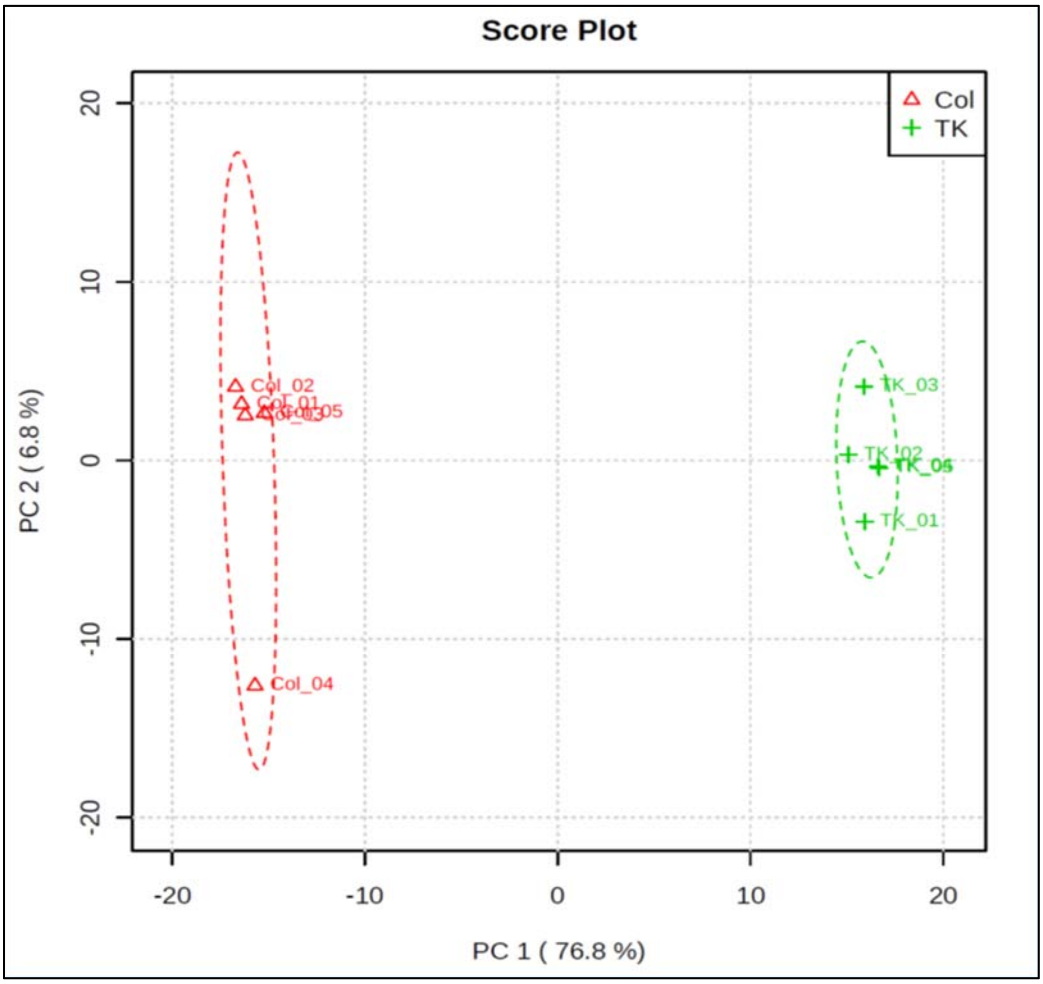

Figure 4.8: Principal component analysis (PCA) of brand (TK) and generic (Col) showing sample clustering. There was clear separation indicating these capsules have a different chemical content

In Figure 4.8, generic (Col) was compared to the reference (TK) using PC1 versus PC2. The five capsule samples derived from generic (Col) clustered together and circled with red line indicating reproducibility between different capsules except for capsule no. 4 which clustered away from main cluster indicating a difference in chemical content from other generic (Col) capsules. On the other hand, the five reference brand (TK) capsules clustered in a smaller cluster compared to (Col) cluster indicating a more homogenous content and better reproducibility between capsules.

Figure 4.9, shows a heatmap and hierarchical clustering for the top 200 metabolomic features that were variably expressed between brand (TK) and generic (Col). The heatmap shows clearly that the top features belong to two different clusters. Some features were present in brand (TK) and absent in generic (Col), whereas others were present in generic (Col) and absent in brand (TK). This confirms the different chemical contents of the two compared drug formulations.

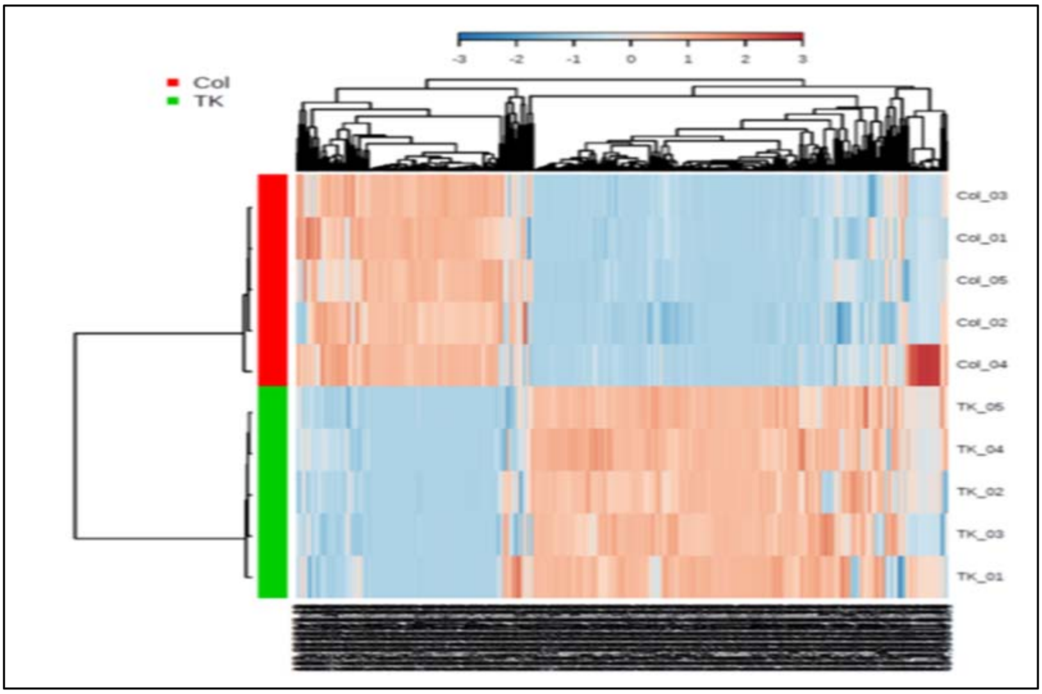

Figure 4.9: Heatmap of brand (TK) and generic (Col) showing hierarchical clustering of top metabolomic features that were variably expressed between brand TK and generic Col.

Table 4.13 shows the preliminary identifications for the impurities found in generic (Col) compared to brand (TK). A 261 fold difference in sorbitol concentrations was detected in the generic capsules ($p<0.0001$).

In addition, contamination with zizyphine A (a plant extract "Ziziphus jujube, Ziziphus vulgaris"), delcorine (an alkaloid, derived during amination reaction in cyclosporin A synthesis) and 2-Oxooctadecanoic acid (an inactive ingredient derived from an oil source) (Kyoto Encyclopedia of Genes and Genomes, 2015) was found. Of note, zizyphine A, is a cyclopeptide product of the central Asian plant Ziziphus oenoplia. Although generic (Col) is a product from South America, however, it is manufactured in central Asia. This specific contaminant confirms the manufacturer origin of the generic (Col) capsules ($p<0.001$), (see appendix 2 for full list of chemical contaminants detected in all generic capsules compared to brand TK).

Table 4.13: Preliminary MS identification of impurities found in generic Col versus brand TK in positive and negative mode

MS-Mode	**M/Z determined monoisotopic mass**	**Theoretical mass***	**Putative ID**	**Fold change**	**P -value**	**Possibilities**
Pos.	612.3741	611.3683	Zizyphine A	3409	6.95E-10	Plant contamination
Pos.	480.2930	479.2883	Delcorine	1077	3.49E-09	Alkaloid derived by amination reaction during CycA synthesis
Neg.	181.0699	182.0790	Sorbitol	261	8.88E-07	Inactive ingredient
Neg.	297.2422	298.2508	2-Oxooctadecanoic acid	26	0.00135	Inactive ingredient

*Theoretical mass data obtained from METLIN: Metabolite Search (https://metlin.scripps.edu/metabo_advanced.php).

Ziziphus is a natural herb used for many aliments and enhancements. Ziziphus produces cyclopeptide alkaloids known as ziziphines and has been used in Chinese herbal medicine and Ayurvedic Indian medicine for many years. It is known to help with relieving stress, purifying the blood, improving the immune system, treating bronchitis, anaemia, irritability, diarrhoea, fatigue, ulcers and even the leaves are used to treat chickenpox, smallpox, measles and many more diseases. It is also used as a weight gainer and to increase muscular strength and endurance. It is often administered when patients experience restless sleeping behaviours, anxiety, reduced memory (forgetfulness) and trouble concentrating (Burton, 2008). Some external uses of the bark can be used to make an eyewash to reduce inflammation (Chen et al., 2015).

4.5 Conclusions

This study represents a simple, rapid, specific and sensitive HPLC method for the determination of an immunosuppressive drug ciclosporin. This method was used successfully to determine of ciclosporin mass amount in brand versus generic capsules.

This study showed that some ciclosporin capsules contain less than the labelled amount. According to the USP a minimum of 80% of labelled amount of ciclosporin should dissolved in 90 min (USP 35, 2012). In this study the ciclosporin capsules from India and Morocco failed to achieve this. Even within the same brand, but from different countries, there were differences in the average content within the acceptable limits. Some ciclosporin products may contain less than the dose required to achieve a therapeutic effect. This may be harmful for patients especially those using NTIDs such as ciclosporin. Irreversible kidney damage could occur at high doses of this drug (Johnston & Holt, 2001) and acute rejection at the lower therapeutic doses. Overall, switching among and between brand and generic ciclosporin can lead to undesirable effects.

Based on the results from the impurity test, we conclude that some of the ciclosporin preparations were found to be contaminated with plant products. This indicates problems with ciclosporin pharmaceutical production, and these could lead to harmful clinical effect on patients.

Chapter 5. Development and application of a UHPLC-MS/MS method for azithromycin

5.1 Introduction

Late in 2013, the FDA launched a warning about a possible fatal side effect of azithromycin. This medication may cause abnormal changes in the electrical activity of the heart. Azithromycin is a macrolide antibiotic belonging to the azalide group. It is used to treat some bacterial infections such as pneumonia, gonorrhoea, sinus and skin. Both brand and generics of azithromycin are available worldwide in different dosage forms.

5.2 Azithromycin

Azithromycin (Sumamed, Zithromax, Zmax) was discovered in 1980 in Croatia by Pliva pharmaceutical company. In 2010, it had become one of the most prescribed antibiotics in United States clinics (Bach and Žubrinić, 2013).

Chemically, azithromycin is described as: (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-11-[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-2-ethyl-3,4,10-trihydroxy-13-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one (Figure 5.1).

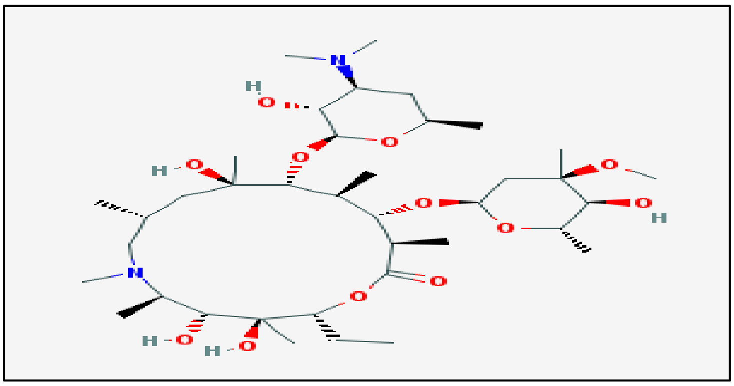

Figure 5.1: Azithromycin chemical structure (National Center for Biotechnology Information, 2005)

5.2.1 Indications

Azithromycin is a widely used antibiotic for treating bacterial infections, caused by bacteria like Streptococcus pneumonia, Haemophilus influenza, Chlamydia pneumonia, Streptococcus pyogenes, Staphylococcus aureus, Neisseria gonorrhoea, which cause respiratory, sinusitis, pharyngitis/tonsillitis, skin and urethritis infections (UpToDate, 2015a).

To ensure effective treatment use of azithromycin, and reduce bacterial resistance, it should only be used when there is a clear indication of a susceptible bacteria or organism (Food and Drug Administration, 2013c).

5.2.2 Mechanism of action

Azithromycin has a bacteriostatic effect. Its inhibits bacterial protein synthesis by binding to the 50S subunit of bacterial ribosomes (Rang et al., 2007).

5.2.3 Absorption

Azithromycin bioavailability is 38%. In a two-way crossover study where 12 healthy test patients were administered a single 500 mg dose of azithromycin (two 250 mg tablets) with or without a high fat meal, food consumption indicated an increase in C_{max} by 23% but had no impact on AUC. With different formulation, azithromycin suspension was given (to test patients) under fed conditions to 28 healthy subjects, C_{max} increased by 56% and there was no change to AUC. Azithromycin C_{max} decreased by 24% when administered with an antacid containing aluminium and magnesium hydroxide, while the AUC did not change (Food and Drug Administration, 2013c).

5.2.4 Distribution

After oral administration, distribution of azithromycin in the body may reach up to 31.1 L/kg. It binds to serum protein in a range from 51% at 0.02 µg/mL to 7% at 2 µg/mL. The concentration of azithromycin is higher in tissues compared to plasma or serum and penetration into the CSF is poor (UpToDate, 2015a).

5.2.5 Elimination

Azithromycin is excreted mainly through the bile as unchanged drug. Approximately 6% of the administered dose appears as unchanged drug in urine (UpToDate, 2015a).

5.2.6 Adverse events (AE)

The common AE of azithromycin include GI disturbances such as diarrhoea, loose stools, abdominal complaints of pain, spasm, flatulence, nausea and vomiting. A reversible increase of some liver enzymes (transaminases, alkaline phosphate) may occur. In blood, neutropenia was noted in some cases. Hypersensitivity reactions of skin and mucosa were rare.

5.2.7 Precautions

Azithromycin should be taken with caution by hepatic disease patients because it is mainly excreted by the liver. There are limited data for patients with renal impairment and azithromycin should be taken with caution. Macrolides have been associated with prolonged QT interval and may cause cardiac arrhythmia, azithromycin treatment should be closely monitored (Lu et al., 2015). Using azithromycin without indication may increase bacterial resistance. There is not enough data about the use of azithromycin and carcinogenesis. There are no confirmed data relating to mutagenic risks and infertility by using azithromycin. There are not enough studies of the effects of using azithromycin in pregnant women. Animal studies showed that there was no direct effect on the foetus and azithromycin is classified under pregnancy category B. Azithromycin should only be administered for pregnant women if a clear requirement is determined. For lactating mothers azithromycin should be used with caution, as it has not been determined whether azithromycin is excreted in milk (Food and Drug Administration, 2013c).

5.2.8 Contraindications

Azithromycin is contraindicated in those with hypersensitivity to azithromycin, other macrolide antibiotics, or any component of the formulation or who have a history of cholestatic jaundice/hepatic dysfunction associated with prior azithromycin use (UpToDate, 2015a).

5.3 QT-interval

The QT interval is the time between depolarization to repolarization of the heart. The QT interval is around 400 ms. Prolongation of the QT interval increases the risk of cardiac arrhythmia which can be fatal (Food and Drug Administration, 2013b, Parnham et al., 2014). The FDA launched a safety announcement about the irregular heart rhythm associated with azithromycin treatment, especially with cardiac disease patients (Food and Drug Administration, 2013b).

5.4 Pharmacodynamics and pharmacokinetics

After oral administration, azithromycin is rapidly absorbed. It distributes in skin, lung, and tonsils. Azithromycin is mainly metabolised by the liver. Additional cation should be considered when treating patients with impaired liver function (Food and Drug Administration, 2013c). The half-life of elimination after oral administration is 68-72 h allowing single dose treatment. Azithromycin is excreted mainly through biliary excretion (UpToDate, 2015a).

5.5 Collection of drug samples (azithromycin)

Nineteen azithromycin products were obtained from hospitals and commercial pharmacies in different countries for inclusion in this study. Four brand samples were manufactured by Pfizer, U.S.A. (Brands A, B, C, and D). Generics (A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O) were manufactured in Africa, Asia, and Europe (Table 5.1).

Table 5.1: Doses and sources of azithromycin tablets

Drug, dose & code	Obtained from	Origin
Azithromycin 500 mg (Brand A)	Commercial pharmacy, USA	Same manufacturer with different batch numbers
Azithromycin 500 mg (Brand B)	Commercial pharmacy, USA	
Azithromycin 500 mg (Brand C)	Commercial pharmacy, USA	
Azithromycin 500 mg (Brand D)	Commercial pharmacy, USA	
Azithromycin 500 mg (Generic A)	Commercial pharmacy, Morocco	Same manufacturer with different batch numbers
Azithromycin 500 mg (Generic B)	Commercial pharmacy, Morocco	
Azithromycin 500 mg (Generic C)	Commercial pharmacy, Morocco	
Azithromycin 500 mg (Generic D)	Commercial pharmacy, Morocco	Same manufacturer with different batch numbers
Azithromycin 500 mg (Generic E)	Commercial pharmacy, Morocco	
Azithromycin 500 mg (Generic F)	Commercial pharmacy, Morocco	
Azithromycin 500 mg (Generic G)	Commercial pharmacy, India	Same manufacturer with different batch numbers
Azithromycin 500 mg (Generic H)	Commercial pharmacy, India	
Azithromycin 500 mg (Generic I)	Commercial pharmacy, India	
Azithromycin 500 mg (Generic J)	Commercial pharmacy, Egypt	Same manufacturer with different batch numbers
Azithromycin 500 mg (Generic K)	Commercial pharmacy, Egypt	
Azithromycin 500 mg (Generic L)	Commercial pharmacy, Jordan	Same manufacturer with different batch numbers
Azithromycin 500 mg (Generic M)	Commercial pharmacy, Jordan	
Azithromycin 500 mg (Generic N)	Commercial pharmacy, India	-
Azithromycin 500 mg (Generic O)	Hospital Pharmacy, United Kingdom	-

5.6 Extraction by dissolution (for azithromycin)

A dissolution method to analyse azithromycin tablets was obtained from the FDA and US Pharmacopeia. The dissolution test was carried out under the following conditions: Temp: 37.5°C ± 0.5, 900 mL 0.1 M phosphate buffer (pH 6.0) was used as a medium, the paddle apparatus (Apparatus 2): 75 rpm, sampling time at (5, 10, 20, 30, 45, and 60 min), with 5 mL volume for each sample. The samples were filtered using 20 µm filters.

There were noticeable differences between brand A and generic A azithromycin tablets specifically in shape (Figure 5.2).

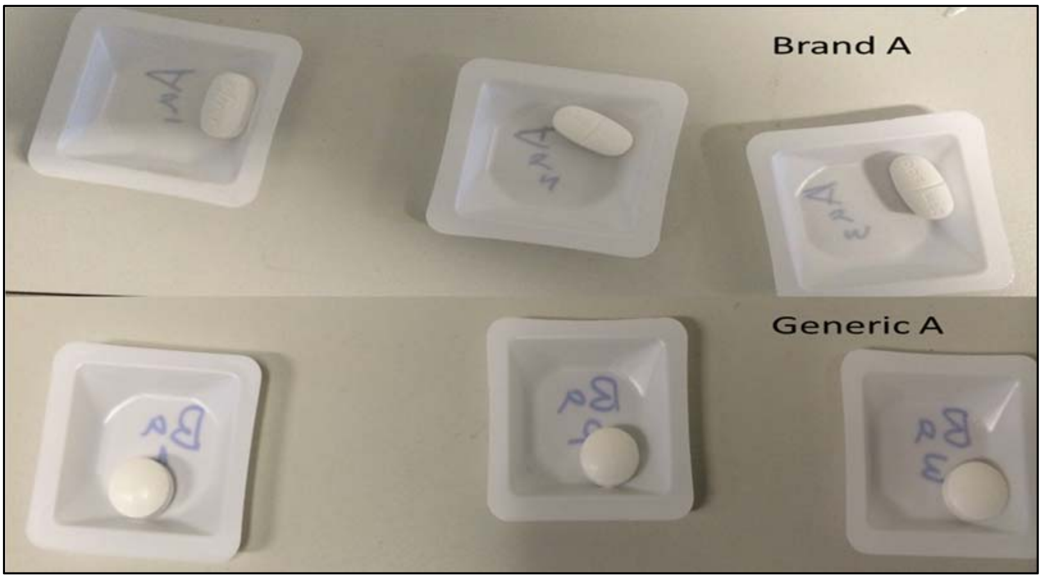

Figure 5.2: The differences in shape between brand A and generic A azithromycin.

During the dissolution test of generic products (D, E, F), they showed different dissolution behaviours compared to the branded counterpart. There was noticeable un-dissolved residue instead of a cloudy solution (Figure 5.3).

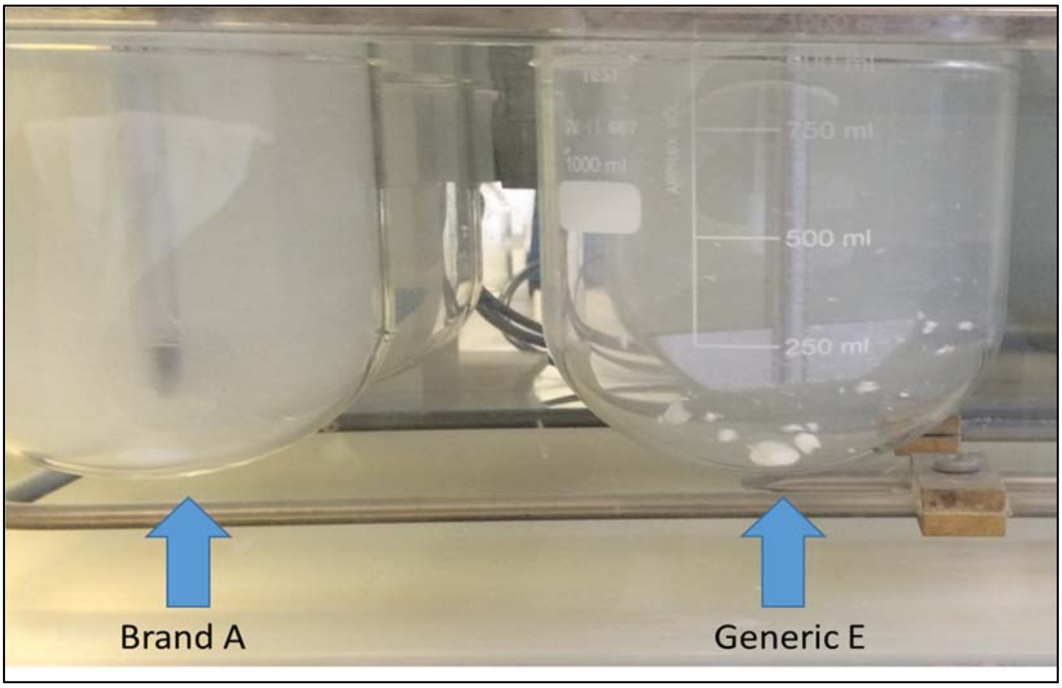

Figure 5.3: Differences in dissolution behaviour between brand A and generic E showing undissolved residue after 30 min.

Moreover, some tablets did not dissolve completely after 30 min (Figure 5.4).

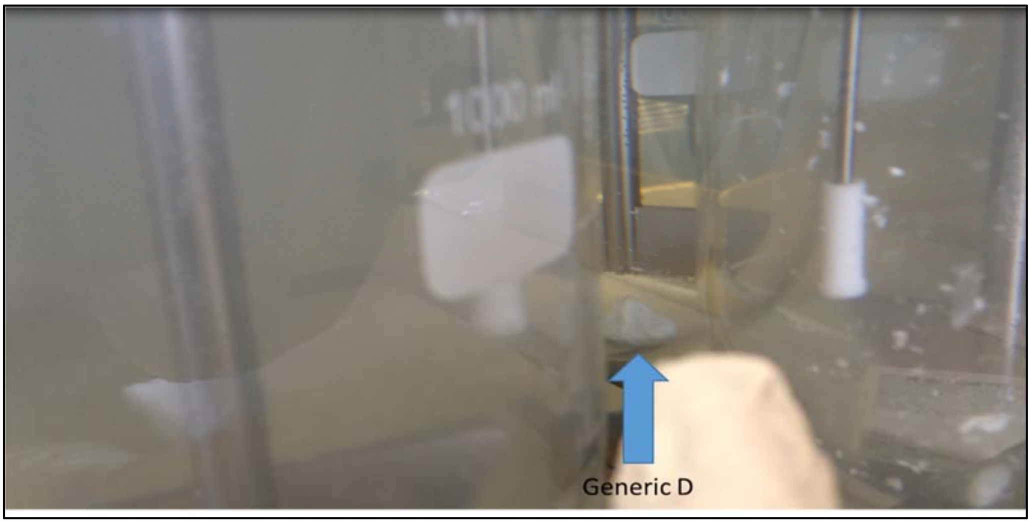

Figure 5.4: Differences in dissolution behaviour, showing undissolved tablet (generic D) after 30 min.

5.7 LC-MS/MS experimental method validation

5.7.1 Optimisation of flow rate

Different flow rates were used to obtain the best resolution and peak shape for azithromycin and its internal standard (roxithromycin).

a) 30 µL/min with 0.1% FA, A: 90 - 5% gradient (Figure 5.5)
b) 30 µL/min without 0.1% FA, A: 90 - 5% gradient (Figure 5.6)
c) 30 µL/min + isocratic 50% A/50% B and without 0.1 %FA (Figure 5.7)
d) 40 µL/min with 0.1% FA, A: 90 - 5% gradient (Figure 5.8)

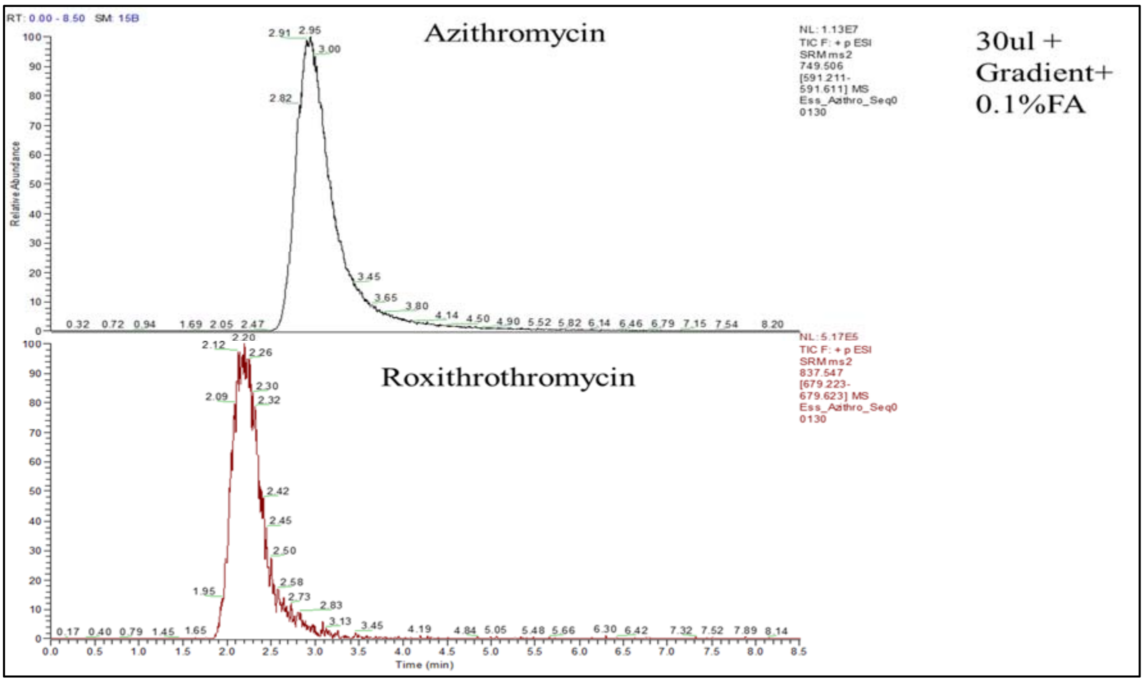

Figure 5.5: Chromatogram showing good detection of azithromycin and IS at 30 µL/min flow rate, with 0.1% FA, A: 90 -5% gradient

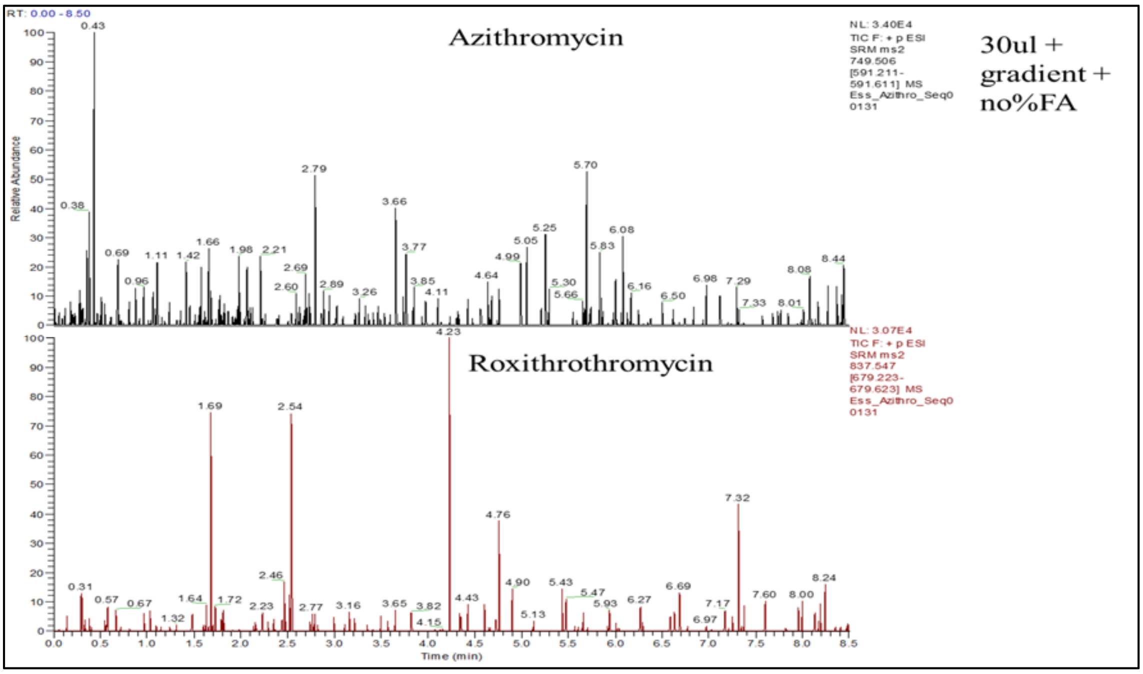

Figure 5.6: Chromatogram showing no detection of azithromycin and IS at 30 µL/min flow rate, with 0 % FA, A: 90 -5% gradient

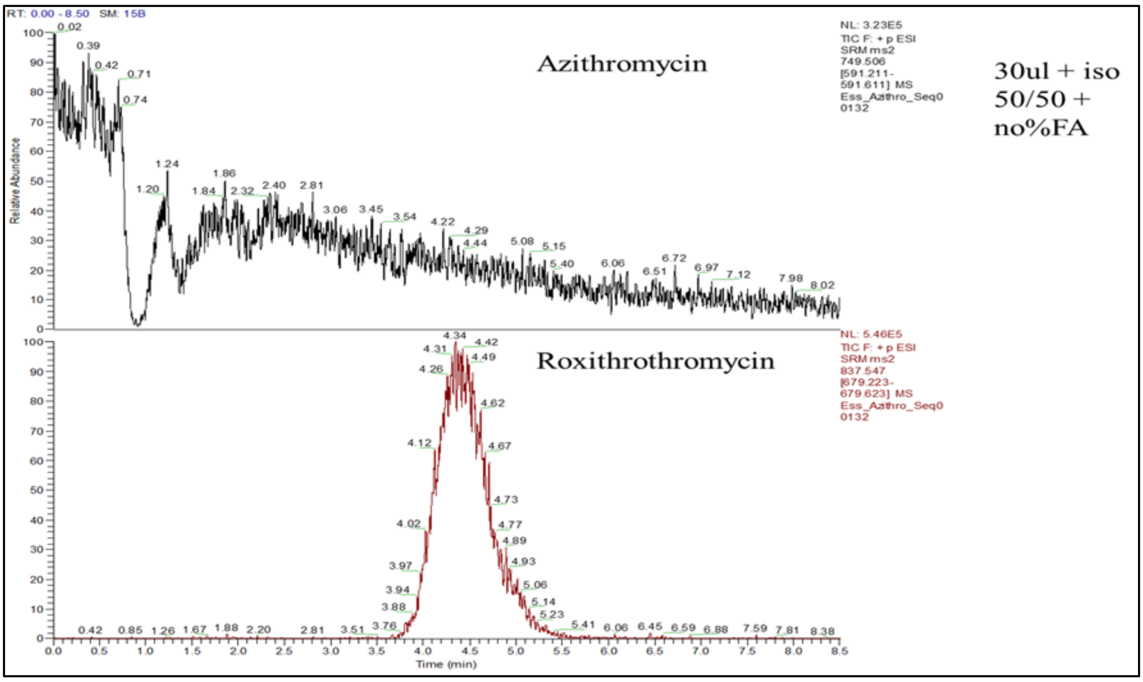

Figure 5.7: Chromatogram showing IS detection and no azithromycin detection at 30 µL/min flow rate, with 0 % FA, isocratic 50% solution A/50% solution B

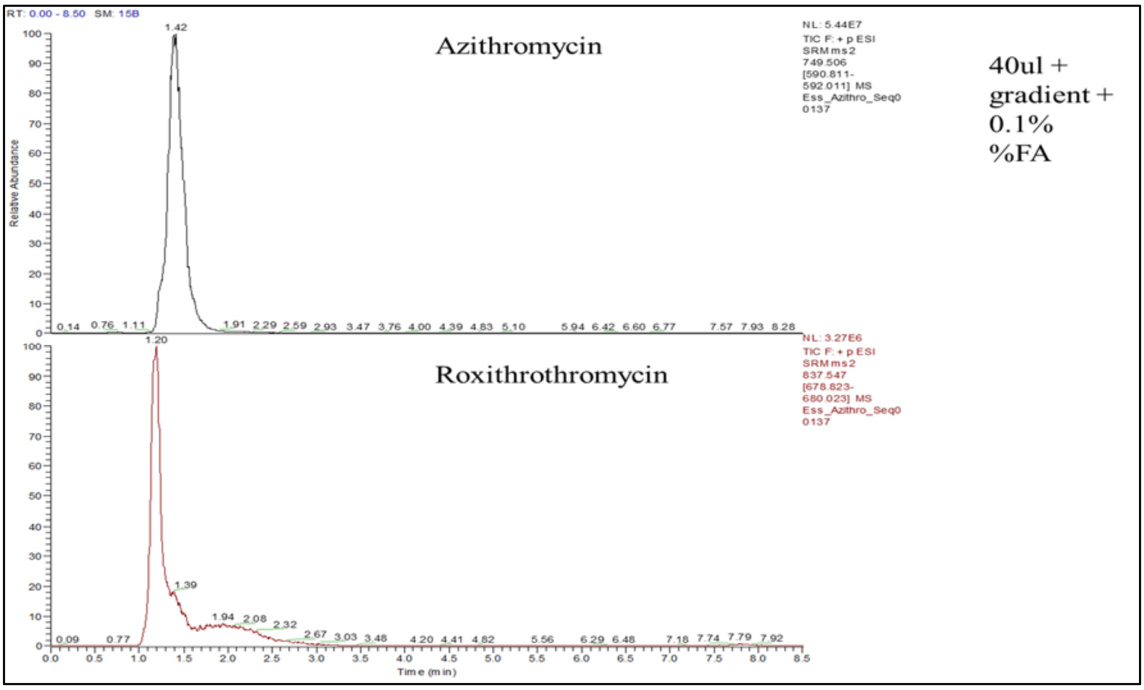

Figure 5.8: Chromatogram showing detection of azithromycin and IS with peak tailing at 40 µL/min flow rate, with 0.1% FA, A: 90 - 5% gradient

The best resolution was found at 30 μL/min in the presence of 0.1% FA. In absence of FA, azithromycin was not detectable indicating that FA is essential for azithromycin ionisation.

5.7.2 Preparation of stock, calibration solutions and control samples

Azithromycin and roxithromycin stock solution (10 mg/mL) was prepared in acetonitrile and stored at 4ºC. The azithromycin calibration standards were freshly prepared at the time of the experiment. Five concentrations of azithromycin (0.01, 0.03 0.1, 0.3, and 1 μg/mL) were prepared in dissolution buffer containing 1 μg/mL roxithromycin.

5.7.3 Calibration and linearity

Calibration standards (0.001–1 μg/mL) were injected into the LC-MS/MS system. The integrated peak areas of azithromycin and internal standard (roxithromycin) were recorded. Linear regression of the standards was done using Microsoft Excel 2010, by plotting the peak area ratio of azithromycin divided by roxithromycin against the azithromycin concentration of the standards. The linearity was checked by calculating r^2 value using least-square linear regression analysis.

Figure 5.9 shows a typical standard curve for azithromycin. Coefficient of correlation was 0.996. The mean slope was 0.2758 ± 0.0044 for azithromycin.

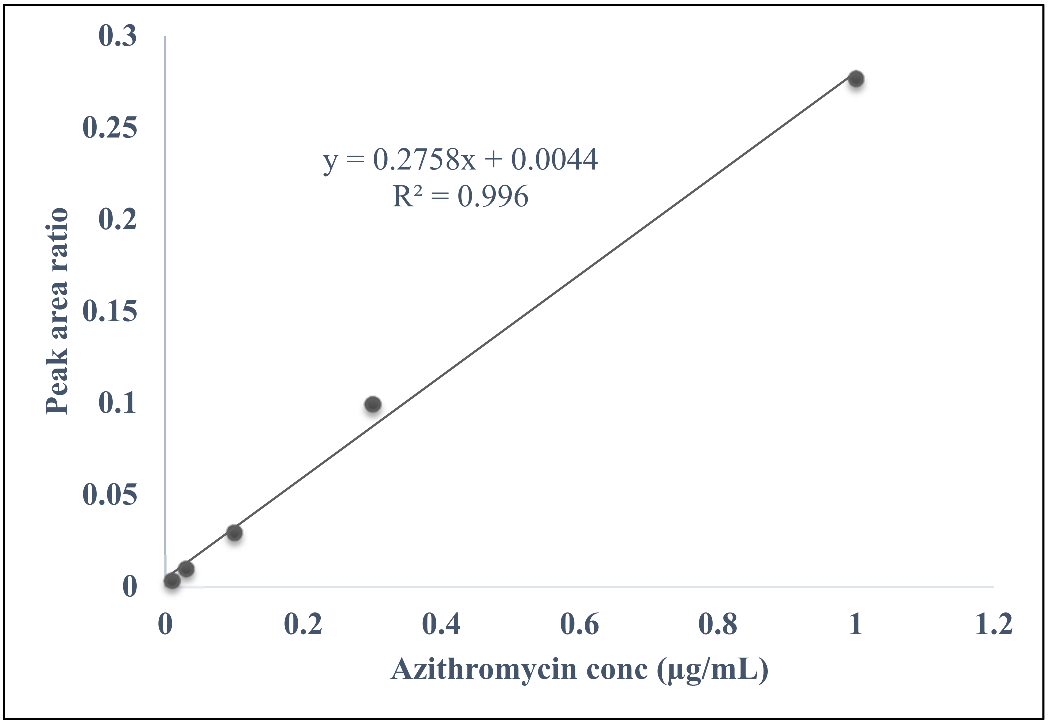

Figure 5.9: Standard curve for azithromycin standards in extraction buffer showing linear calibration line $r^2 = 1$ using the UHPLC-MS/MS system

5.7.4 Method sensitivity

The lower limit of detection (LLOD) of azithromycin is the lowest concentration with a peak area ratio three times higher than the baseline noise. The lower limit of quantification (LLOQ) was determined as the lowest concentration of azithromycin with a peak area ratio five times higher than the baseline noise with imprecision <10% and inaccuracy <15% (Table 5.2).

Table 5.2: Sensitivity test of azithromycin standard showing the concentration of LLOD and LLOQ on different days

Days	LLOD (0.001 µg/mL)	LLOQ (0.01 µg/mL)
1	0.0007	0.01
2	0.0013	0.01
3	0.0013	0.01
4	0.0016	0.01
5	0.0013	0.01
Average	0.0012	0.01
±SD	0.0004	0.0005
CV%	24.70%	6.21%
Inaccuracy	36.8%	10.6%

The LLOD was found to be 0.001 µg/mL with a CV% of 24% and inaccuracy of 37%. As these values were not acceptable, this calibration level was removed from the standard curve. The LLOQ was found to be 0.01 µg/mL with a CV% of 6.2% and inaccuracy of 10.6%.

5.7.5 Method specificity

The method specificity was investigated by injecting blank extraction buffer before the start of each run. The blank standards were used to control for any interfering peaks that elute at the same retention time as azithromycin and roxithromycin method was specific. There were no significant interfering peaks before, between and after each run (Figure 5.10).

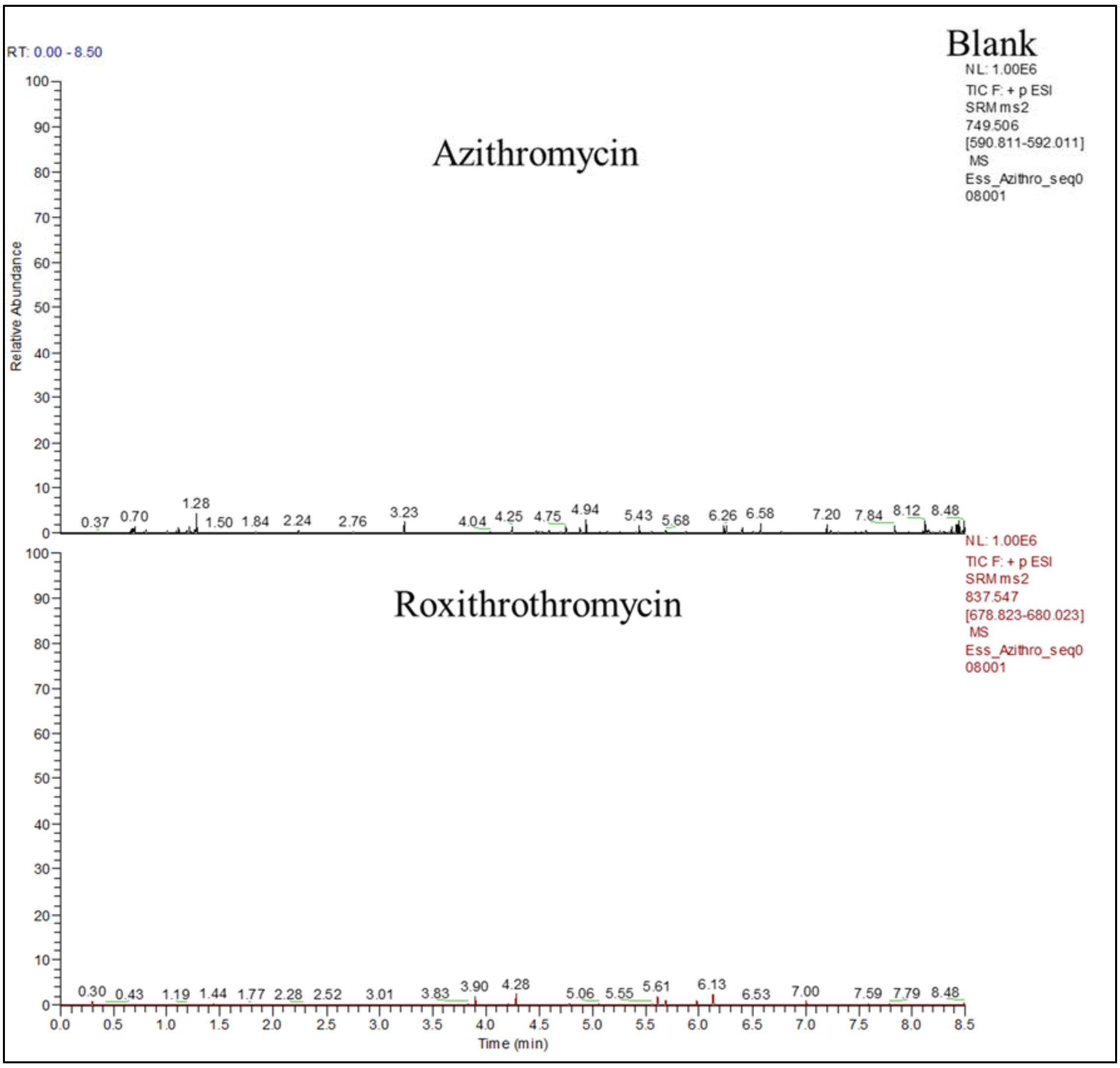

Figure 5.10: Example chromatogram to show there are no interfering peaks before running azithromycin samples

5.7.1 Imprecision

5.7.1.1 Inter-day variability

Quality control samples (QC) of 0.05 and 0.5 µg/mL were prepared by dilution of azithromycin stock in extraction buffer solution.

Imprecision was determined by calculating the coefficient of variation (CV %) of the quality control (QC) samples using the following equation.

$$CV\% = \frac{\text{standard deviation of QC concentration}}{\text{average of the QC concentration}} \times 100$$

Equation 4: Calculation of coefficient of variation to check inter-day variability of azithromycin

Azithromycin known concentration 0.05 µg/mL (low quality control; LQC) and 0.5 µg/mL (high quality control; HQC) were injected into the LC-MS/MS system. Table 5.3 shows the hypothetical and measured concentration by LC-MS/MS analysis for these samples over five different days along with the average, standard deviation, and the coefficient of variation percentage in order to determine the interday imprecision.

Table 5.3: The average, standard deviation (±SD) and the coefficient of variation (CV) for the inter-day variability for azithromycin assay standards at 0.05 and 0.5 µg/mL

Conc. (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV%**
0.5	0.51	0.48	0.54	0.46	0.45	0.5	0.0343	0.91%
0.05	0.051	0.049	0.063	0.051	0.055	0.057	0.0043	7.58%

Interday imprecision for both LQC and HQC were acceptable (< 8%) indicating good reproducibility between days.

5.7.1.2 Intraday variability

Azithromycin LQC and HQC were injected into the LC-MS/MS system. Table 5.4 shows the hypothetical and measured concentration by LC-MS/MS analysis for five different samples within the same day along with the average, standard deviation, and the coefficient of variation percentage in order to determine the intraday imprecision.

Table 5.4: The average, standard deviation (±SD) and the coefficient of variation (CV) for the intraday variability for azithromycin assay standards at 0.05 and 0.5 µg/mL

Conc. (µg/mL)	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Mean**	**SD**	**CV%**
0.5	0.44	0.45	0.52	0.47	0.49	0.47	0.0333	7.05%
0.05	0.054	0.049	0.061	0.057	0.054	0.055	0.0042	7.58%

Intraday imprecision was determined by extraction and analysis of 5 replicates of each of the two QC samples within the same run.

Intraday imprecision for both LQC and HQC was acceptable (< 8%) indicating good reproducibility within day.

5.7.2 Inaccuracy

The inaccuracy of the assay was determined by measuring the difference between actual (hypothetical) and measured concentration of each azithromycin calibration standard. The concentration difference was then divided by the actual concentration and multiplied by 100 (Equation 5).

$$\text{Inaccuracy} = \frac{\text{Actual conc. - measured conc.}}{\text{Actual conc.}} \times 100$$

Equation 5: Inaccuracy percentage calculations of hypothetical and measured azithromycin concentrations

Table 5.5: Inaccuracy of the assay was < 13% of the true value at standard curve concentrations ranging from 0.01 to 1 µg/mL of azithromycin

Conc. (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Average Conc. µg/mL**	**Inaccuracy**
0.01	0.01	0.01	0.01	0.01	0.01	0.01	9.6%
0.03	0.03	0.03	0.04	0.03	0.03	0.03	12.2%
0.1	0.09	0.11	0.11	0.10	0.11	0.10	4.1%
0.3	0.32	0.33	0.30	0.30	0.30	0.31	3.4%
1	0.88	0.92	0.87	0.84	0.85	0.87	-12.6%

The inaccuracy values were acceptable (<15%) for all of the five azithromycin standards (Table 5.5).

5.7.3 LC-MS/MS system carryover

The LC-MS/MS system carryover was checked by running blank samples between actual samples. The blank sample was checked for the presence of any carried over azithromycin and roxithromycin. Calibration standards were also randomised and the calibration curve linearity was then checked in order to detect any carryover (Figure 5.11).

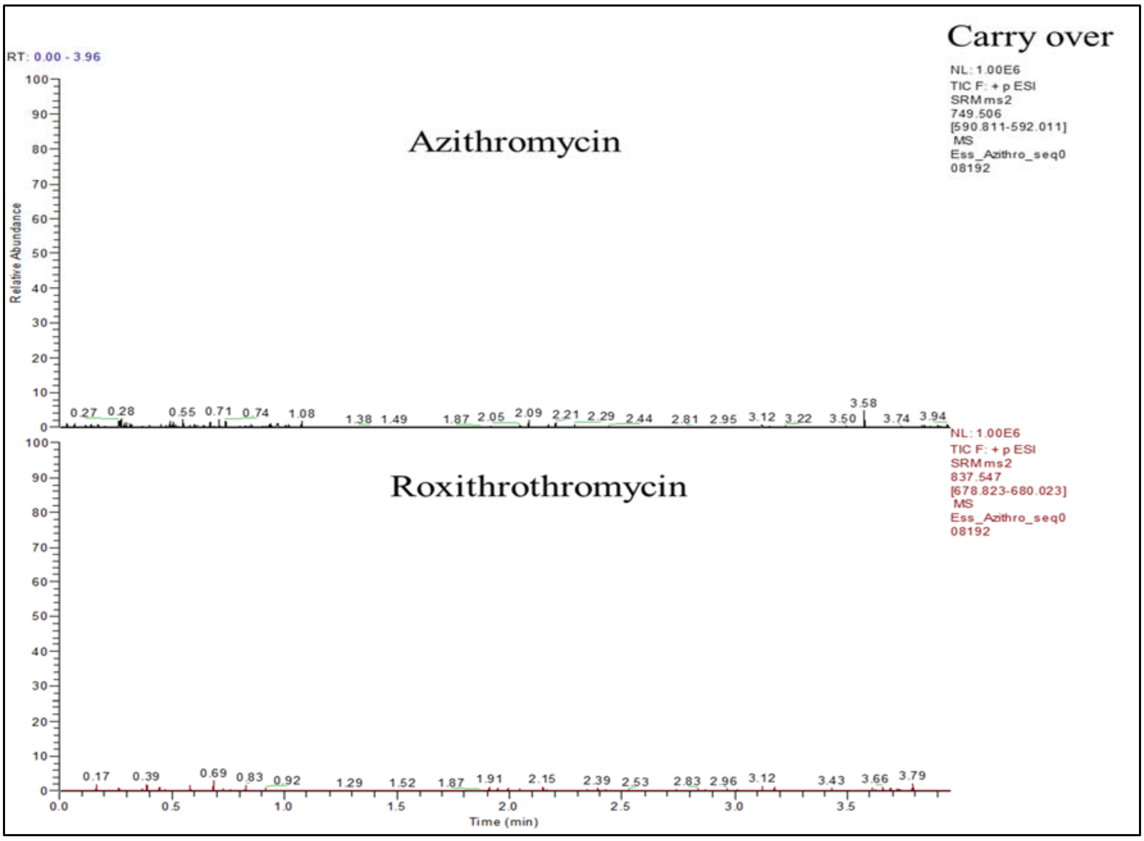

Figure 5.11: A blank run after actual sample showing no carry over between samples

5.7.4 Statistical analysis and graphic presentation

The graphic presentations and the statistical analyses were done using Excel 2010 for Windows version, 14.0.6112.500 (32-bit) and Minitab version, 16.

5.7.5 Dissolution extraction recovery

The recovery of the volume was calculated by dividing the actual volume over the total volume then multiplied by the concentration (Table 5.6).

$$\text{Recovery} = \frac{\text{Actual volume}}{\text{Total volume}} \times \text{Conc.}$$

Equation 6: Recovery calculation of azithromycin standards

Table 5.6: The average recovery percentage of azithromycin mass amount after 30 and 60 min and standard error of the dissolution test (n = 3)

Recovery average		
Azithromycin	**Av. Content% 30 min**	**Av. Content% 60 min**
Brand A	91% ±9.9	100% ±6.3
Brand B	94% ±4.8	88% ±2.2
Brand C	90% ±1.4	96% ±5.3
Brand D	99% ±6.4	91% ±3
Generic A	82% ±4.3	77% ±3.6
Generic B	87% ±13.8	80% ±6.7
Generic C	86% ±6.2	87% ±5.5
Generic D	32% ±17.5	58% ±19.6
Generic E	71% ±8.4	106% ±4.1
Generic F	71% ±9.3	118% ±11.3
Generic G	107% ±14.6	104% ±5.8
Generic H	62% ±10.7	61% ±7.8
Generic I	88% ±13.1	118% ±5.1
Generic J	96% ±1.3	84% ±6.2
Generic K	115% ±5.8	97% ±7.1
Generic L	94% ±4.5	84% ±2.5
Generic M	106% ±0.97	99% ±10.3
Generic N	45% ±19.1	63% ±6.8
Generic O	86% ±5.7	62% ±3.4

This test was done in order to confirm that the dissolution test successfully recovered all the tablet content after 30 and 60 min.

5.7.6 Stability

Freeze thaw stability was done for three azithromycin calibration standards stored at -20°C, thawed and re-frozen every week for three weeks.

This test was done to check azithromycin standards stability in stock solutions. Azithromycin standards were stable for the whole period of the experiment. Two different concentrations of 0.03 and 0.3 µg/mL from week 1, 2, and 3 were measured (Table 5.7).

The azithromycin standards were stable for freeze thaw cycles. Average measured concentration for 3 cycles of 0.03 and 0.3 µg/mL of azithromycin was 0.033 and 0.32, respectively. The CV% of 0.03 and 0.3 µg/mL of azithromycin standards were less than 6%. Inaccuracy of the measured concentration was less than ±12%.

Table 5.7: The average concentration (±SD) and coefficient of variation (CV) and inaccuracy of azithromycin stability in stock solution for week 1, 2, and 3

Conc. µg/mL	Week 1	Week 2	Week 3	Average	±SD	CV	Diff.	Inaccuracy
0.03	0.03	0.03	0.03	0.03	0.001	3.66%	0.003	11.6%
0.3	0.3	0.3	0.3	0.3	0.019	5.76%	0.022	7.5%

5.7.7 Final results

The method was successfully applied to measure the actual concentration in each azithromycin product using LC-MS/MS analysis. Sampling time was done at six different intervals in order to check for azithromycin release from tablets. All branded products from Pfizer (A, B, C, D) and ten generics from different manufacturers (A, B, C, G, I, J, K, L, M, O) showed more than 80% of labelled amount in azithromycin capsules after 30 min of the dissolution test giving 91 ± 9.9, 94 ± 4.8, 90 ± 1.4, 99 ± 6.4, 82 ± 4.3, 87 ± 13.8, 115 ± 5.8, 86 ± 6.2, 107 ± 14.6, 88 ± 13.1, 96 ± 1.3, 94 ± 4.5, 106 ± 0.97 and 86 ± 5.7% (±SD), respectively. Five generics (D, E, F, H, N), showed less than the minimum percentage of labelled amount (80% after 30 min) 32 ± 17.5, 71 ± 8.4, 71 ± 9.3, 62 ± 10.7 and 45 ± 19.1% respectively, (Figure 5.12). Relative to brand (A), statistical analysis showed significant differences ($p<0.0001$) of the mean percentage content between brand and generic, the 95%

CI range for the brands (B, C, D) were (74-115), (84-96) (71-127), respectively, and (63-100), (27-146), (60-113), (43-107), (35-107), (31-111), (44-170), (16-108), (32-144), (90-101), (90-140), (74-114), (102-110), (37-127), (62-110) for the generics (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O), respectively (Table 5.8).

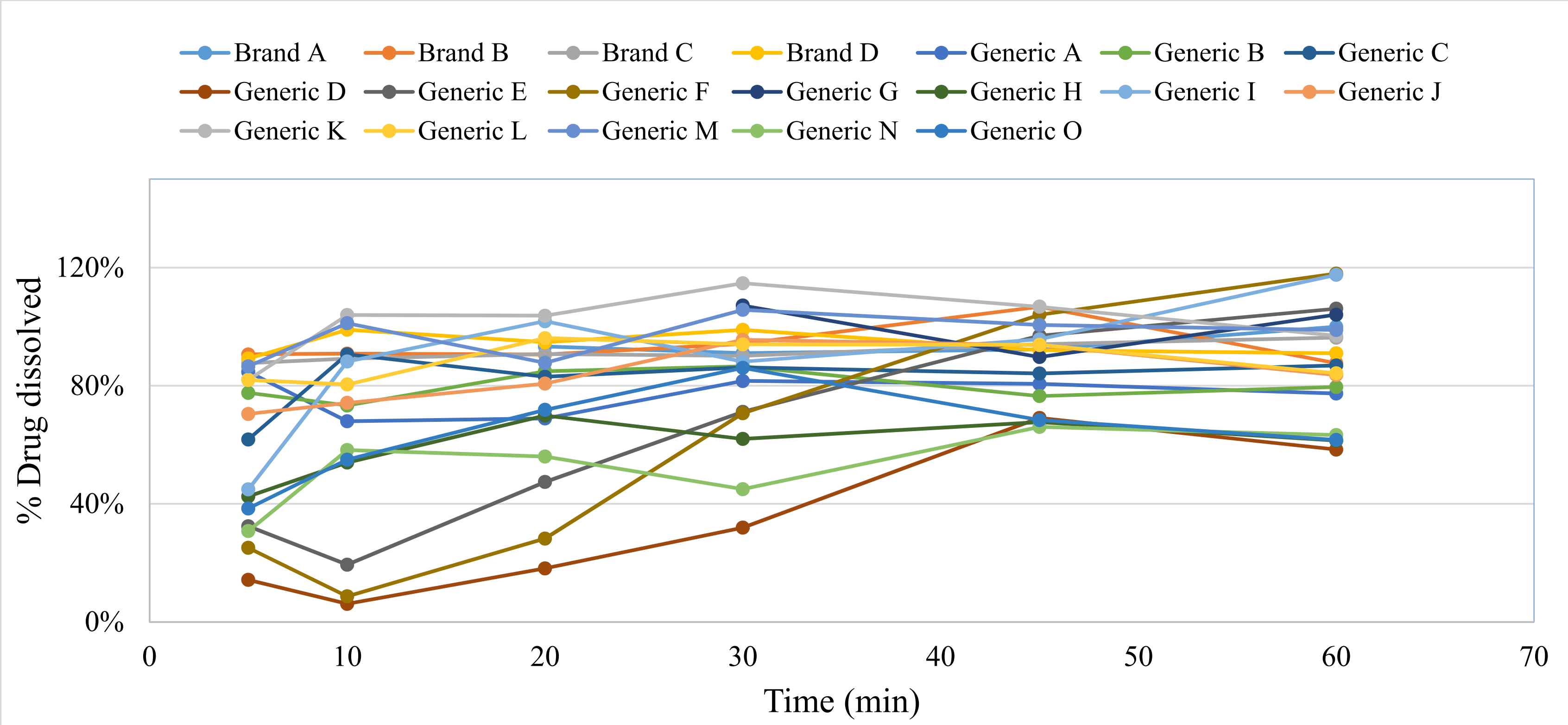

Figure 5.12: Percentage dissolved azithromycin in brand and generics (n = 3)

Table 5.8: The average percentage of azithromycin mass amount, standard deviation, coefficient of variation and 95% CI based on reference capsule (Brand A), (n = 3)

Drug	N	% of mass amount at 30 min (mg/mL)	± SD	CV (%)	95% confidence interval
Brand B	3	94	4.8	9	(74-115)
Brand C	3	90	1.4	3	(84-96)
Brand D	3	99	6.4	11	(71-127)
Generic A	3	82	4.3	9	(63-100)
Generic B	3	87	13.8	28	(27-146)
Generic C	3	86	6.2	13	(60-113)
Generic D	3	32	17.5	95	(43-107)
Generic E	3	71	8.4	21	(35-107)
Generic F	3	71	9.3	23	(31-111)
Generic G	3	107	14.6	24	(44-170)
Generic H	3	62	10.7	30	(16-108)
Generic I	3	88	13.1	26	(32-144)
Generic J	3	96	1.3	2	(90-101)
Generic K	3	115	5.8	9	(90-140)
Generic L	3	94	4.5	8	(74-114)
Generic M	3	106	0.97	2	(102-110)
Generic N	3	45	19.1	74	(37-127)
Generic O	3	86	5.7	11	(62-110)

5.8 Conclusions

All branded products from Pfizer (A, B, C and D) met the USP requirement. Some generic products made by one manufacturer from different batch numbers met the USP requirement, generic (A, B and C). Another generic manufacturer with different batch numbers, generic (D, E and F), failed to meet the USP requirement. However, after 60 min, for two products (generic E and F) more than 80% of azithromycin dissolved. This highlights differences in dissolution behaviours between branded and generic copies, which is a delay in the dissolution rate. For generic (G, H and I), an interesting observation occurred. Two out of three products (G and I) met the USP requirement and one (generic H) failed. Generic products (J, K, L, M and O) met the USP requirement and generic product (N) failed. The causes for dissolution test failure are not clear, but it could be due to bad storage conditions, lack of GMP and/or GLP, resulting in substandard products.

As shown in the dissolution test, some generic products (Generic D, E and F) had different dissolution behaviours compared to the branded counterpart. There was noticeable undissolved residue instead of a cloudy solution and some tablets did not dissolve completely (Figure 5.3 and figure 5.4). Therefore, it could be the main reason why they failed to meet the USP requirements and showed poor dissolution profiles (average content 32, 71, and 71% after 30 min).

Chapter 6. Bioequivalence study of two azithromycin products after oral administration in healthy adults under fasting conditions

6.1 Introduction

This study was performed to investigate the bioequivalence of azithromycin between test product Mazit capsules 250 mg (250 mg azithromycin per capsule: Neopharma, UAE), and reference product Zithromax™ (250 mg azithromycin per capsule, Pfizer Italia, S.R.L., Italy) in fasting healthy subjects.

The study was conducted in collaboration with Neopharma Pharmaceutical Company in United Arab Emirates. The study was a single dose, open label, randomised, two-way crossover design. Clinical investigation of the study was conducted in the International Pharmaceutical Research Centre (IPRC), Amman, Jordan, according to the International Conference on Harmonisation, ICH for Good Clinical Practice (GCP) guidelines, adopted by the European Medicines Agency (EMA). The study protocol called for the 24 healthy volunteers plus 1 to 4 alternates. Demographic data and all clinical assessment along with laboratory evaluation were performed for all enrolled subjects.

The subjects received two capsules of Mazit Capsules 250 mg (250 mg azithromycin per capsule) and two capsules of Zithromax™ (250 mg azithromycin per capsule) in a randomised fashion with a washout period of 21 days. Twenty-eight healthy volunteers enrolled and completed the crossover. The bioanalysis of clinical plasma samples was accomplished by LC/MS/MS detection, which was developed, and validated in accordance with international guidelines at the IPRC. Pharmacokinetic parameters were determined by standard non-compartmental methods, and ANOVA statistics were calculated using Microsoft Excel 2013 and Minitab-17 Statistical Software. The 90% confidence intervals for the ratio (or difference) between the test and reference product pharmacokinetic parameters of C_{max}, t_{max} and AUC_{0-72} were calculated.

6.2 Aims and Objectives

The aim of this study was to investigate the bioequivalence between the test product, Mazit capsules 250 mg, Neopharma, UAE, and its reference product Zithromax™, 250 mg, Pfizer Italia, S.R.L., Italy, in fasting healthy subjects.

6.3 Subjects

Test subjects were recruited from Amman, Jordan and the surrounding areas. Table 6.1, showing the demographic data and sequence of participating test subjects. For participation in the study, test subjects had to meet the selection criteria outlined in the study protocol. Volunteers were informed, by the IPRC representatives, about the aim of the study and any potential risks. Volunteers signed written informed consent (IC) statements, and they were free to withdraw at any time during the course of the study. The investigators had the right to exclude or discontinue any test subjects if they felt that for any reason it would be better to do so, such as risks to their health or signs of side effects or failure to abide by the study protocol and its requirements.

Table 6.1: Demographic data and individual values for the eligible 24 male subjects for the bioequivalence study between Mazit Capsules 250 mg (Neopharma, UAE) and Zithromax™ manufactured by (Pfizer Italia S.R.L., Italy)

Subject No.	**Sequence**	**Height (cm)**	**Weight (kg)**	**Age (years)**
1	BA*	165	80	31
2	AB	171	60	26
3	AB	170	66	22
4	BA	178	60	24
5	AB	188	90	32
6	BA	178	65	31
7	BA	160	65	34
8	BA	172	70	28
9	BA	187	88	30
10	AB	182	85	24
11	AB	170	65	22
12	AB	177	60	23
13	AB	162	60	37
14	AB	172	70	28
15	AB	167	70	25
16	AB	171	70	36
17	BA	181	85	24
18	BA	170	82	26
19	BA	175	60	30
20	AB	175	65	25
21	BA	176	75	20
22	BA	181	70	28
23	AB	174	80	25
24	BA	180	80	22
N		24	24	24
Mean		174	72	27
SD		7.08	9.76	4.61
CV%		4.06	13.60	16.93
SEM		1.44	1.99	0.94
Minimum		160	60	20
Median		175	70	26
Maximum		188	90	37

*A: Test product, (Mazit), B: Reference product (Zithromax™).

The subjects age ranged between 20 - 37 years (27 ± 4.61 years). Weight at screening was between 60-90 kg (72 ± 9.76 kg). Height was between 160 - 188 cm (174 ± 7.08 cm).

Each subject received a thorough physical assessment, vital signs evaluation (blood pressure, pulse, respiratory rate and temperature) and ECG on screening examination. The subjects received the same physical assessment and vital signs evaluation and ECG plus liver function test on follow up examination, which was within at least 24 h from collecting the last sample in period 2.

6.3.1 Inclusion criteria

To be eligible for participation in the study, subjects were examined according to following criteria before their enrolment in the study.

- Age 18 – 45 years, inclusive.
- Subject does not having known allergy to the drug under investigation (azithromycin) or any of its other ingredients or any related drug (erythromycin, any macrolide or ketolide antibiotic).
- Medical demographics are within the normal range performed not longer than two weeks before the initiation of the clinical study.
- Results of laboratory tests are within the normal range. (Laboratory tests are performed not longer than two weeks before the initiation of the clinical study).
- Body weight within 15% of ideal weight for height (Table of “Desirable Weights of Adults”, Metropolitan Life Insurance Company Statistical Bulletin, 1983).

6.3.2 Exclusion criteria

- History of drug or alcohol abuse.
- Acute infection within one week preceding first study drug administration.
- Medical demographics with deviations from reference ranges.
- Subject does not agree not to consume any beverage or food containing methyl-xanthines e.g. caffeine (coffee, tea, energy drinks, chocolate etc.) 24 h prior to the study drug administration of either study period until the end of confinement.

- Subject is a heavy smoker (more than 10 cigarettes per day).
- Subject does not agree not to take any prescription or non-prescription drugs within two weeks before first study drug administration and until the end of the study.
- Subject does not agree not to take any vitamins taken for nutritional purposes within two days before first study drug administration and until the end of the study.
- Results of laboratory tests, which are outside the reference ranges.
- Subject is on a special diet (for example subject is vegetarian).
- Subject consumes large quantities of alcohol or beverages containing methyl-xanthines e.g. coffee, tea, energy drinks, chocolate etc.).
- Subject does not agree not to consume any beverages of food containing alcohol 48 h prior to study drug administration until donating the last sample in each respective period.
- Subject does not agree not to consume any beverages or food containing grapefruit seven days prior to first study drug administration until the end of the study.
- Subject has a history of severe diseases, which have direct impact on the study.
- Participation in a bioequivalence study or any clinical study within the last two months before first study drug administration.
- Subject intends to be hospitalized within three months after the first study drug administration.
- Subjects who through completion of the study would have donated more than 500 mL of blood in 14 days or 750 mL of blood in 30 days, 1000 mL in 90 days, 1250 mL in 120 days, 1500 mL in 180 days, 2000 mL in 270 days, 2500 mL of blood in 1 year.
- The subject is a pregnant female (positive urine or blood pregnancy test) or a lactating female in case there were female participants (no applicable as only men were used in this particular study but still an applicable exclusion criteria if women were included as part of any other current or future study).
- Subject has a history or presence of significant asthma, peptic or gastric ulcer, sinusitis, pharyngitis, renal disorder, hepatic disorder, cardiovascular disorder, neurological disease, haematological disorders or diabetes, psychiatric, dermatologic or immunological disorders.

- Subject who has been engaged in strenuous exercise at least one day prior to dosing till the last sample of each respective period.
- Subject is taking one or more of the following medications, nelfinavir, warfarin, atorvastatin, carbamazepine, cetirizine, didanosine, efavirenz, fluconazole, indinavir, midazolam, rifabutin, sildenafil, theophylline (intravenous and oral), triazolam, trimethoprim/sulfamethoxazole or zidovudine, digoxin, ergotamine or dihydroergotamine, terfanadine, ciclosporin, hexobarbital and phenytoin, antacids containing aluminium and magnesium hydroxide, cimetidine.
- Subjects who have been diagnosed with liver disease, kidney disease, certain heart problems (abnormalities ECG, slow heartbeat, heart failure), family history of certain heart problems, pneumonia, colitis, fungal infection.
- Subject has a history of difficulties in swallowing or any gastrointestinal disease, which could affect the drug absorption.

Forty eight healthy subjects were recruited according to the selection criteria described in the study protocol and volunteered for participation in the study (Figure 6.1). All participating subjects were treated as a single group. Each subject was examined thoroughly during the screening procedure as described in the study protocol (the screening time been set to be not more than two weeks prior to the first study drug administration of study period 1).

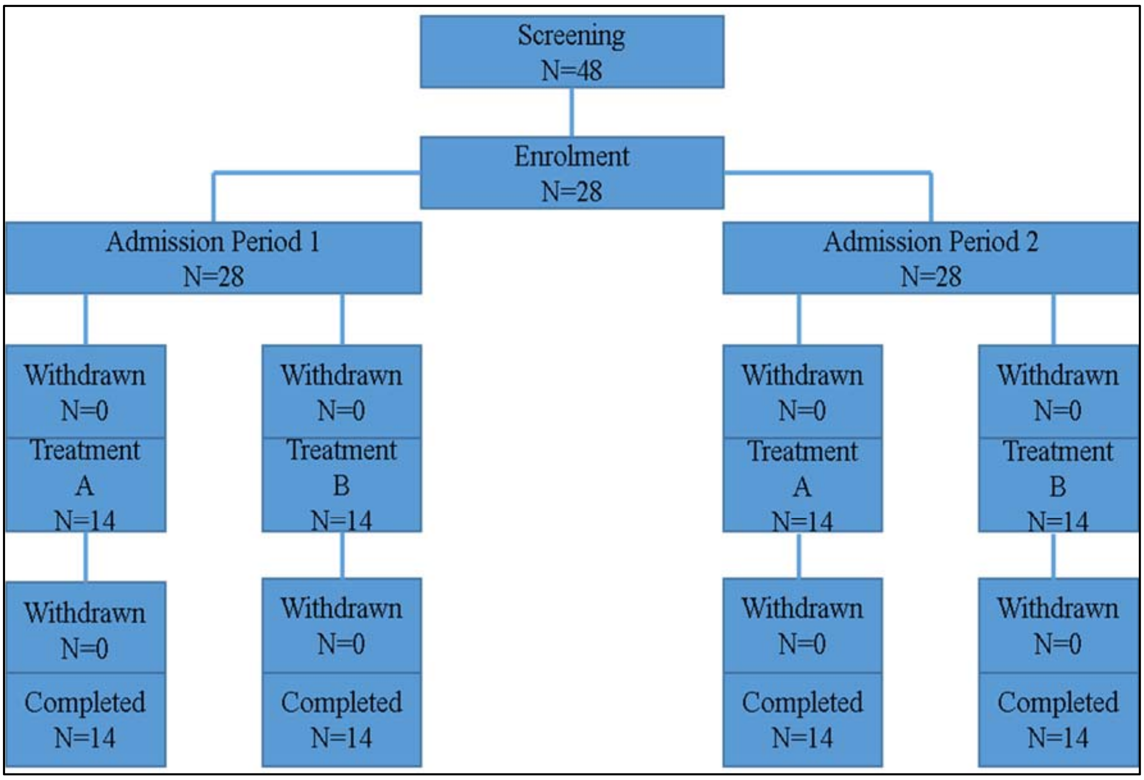

Figure 6.1: Disposition of subjects

6.3.3 Subject identification

During the screening process only their initials identified all test subjects. Each test subject on admission for period 1 was assigned numbers in sequential order. Test subjects throughout the duration of the study retained their represented identification numbers. For data processing and reporting, their assigned identification numbers and initials only identified test subjects.

6.3.4 Withdrawal and exclusions

During this study, 48 subjects were screened. six subjects withdrew for abnormal laboratory results, 12 subjects withdrew for personal reasons and 2 subjects withdrew for medical conditions. A total of 28 subjects were enrolled and completed the crossover. Four subjects experienced vomiting during the bioequivalence study for unknown reasons and were excluded from statistical analysis. Subject withdrawal can be divided into 3 groups (Table 6.2). For more details, see (Appendix 5).

Table 6.2: Types of subjects withdraw from the study

Types of Withdrawal	**Definition**
Withdrawal Group 1	Withdrawal after screening procedures have been performed but before study drug administration in study period 1
Withdrawal Group 2	Withdrawal after study drug administration in study period 1 but before study drug administration in study period 2
Withdrawal Group 3	Withdrawal after study drug administration in study period 2

6.4 Randomisation

The study was a randomised two-way, two sequence crossover design. The order in which the test and reference medicines were received by each test subject was determined by the randomisation plan (Table 6.3). Test subjects were assigned a number in sequential order as per the arrival sequence numbers provided on the test subjects arrival at the centre, by check in for period one and based on adherence of the determined protocol requirements.

All clinical data from this study were collated in case report forms (CRF's) by members of staff in the IPRC. The principal investigators, to ensure correct and accurate completion, evaluated all case report forms. The randomisation codes were withheld from the study personal (see Appendix 7).

Table 6.3: Randomisation plan for the bioequivalence study between Mazit capsules 250 mg, (250 mg azithromycin per capsule) and Zithromax™, (250 mg azithromycin per capsule)

Subject number	**Treatment**	
	Study period 1	**Study period 2**
1	B	A
2	A	B
3	A	B
4	B	A
5	A	B
6	B	A
7	B	A
8	B	A
9	B	A
10	A	B
11	A	B
12	A	B
13	A	B
14	A	B
15	A	B
16	A	B
17	B	A
18	B	A
19	B	A
20	A	B
21	B	A
22	B	A
23	A	B
24	B	A
25	A	B
26	A	B
27	B	A
28	B	A

A: The test product, Mazit, Neopharma, 250 mg azithromycin per capsule

B: The reference product, Zithromax™, Pfizer Italia, S.R.L., Italy., 250 mg azithromycin per capsule.

6.5 Study drug administration

Study drugs were administered by the clinical staff of the IPRC as follows:

- Treatment A: Two capsules of Mazit capsules 250 mg, test product, 250 mg azithromycin per capsule were given with 240 ml of water. Water was at room temperature and was measured with a 250 ml cylinder.
- Treatment B: Two capsules of Zithromax™, reference product, 250 mg azithromycin per capsule were given with 240 ml of water. Water was at room temperature and was measured with a 250 ml cylinder (Table 6.4).

Table 6.4: Identity of the study medications involved in the bioequivalence study between Mazit capsules 250 mg (Neopharma, UAE) and Zithromax™ (Manufactured by Pfizer Italia S.R.L., Italy).

Identification	**Test product- Treatment A**	**Reference product-Treatment B**
Brand name	Mazit Capsules 250 mg	Zithromax™
Dosage form	Hard Gelatine Capsule	Hard Gelatine Capsule
Strength	250 mg azithromycin	250 mg azithromycin
Manufacturer	Neopharma, UAE	Pfizer Italia S.R.L. Italy
Batch No.	AZA8002	86408002
Expiry Date	09/2010	03/2013

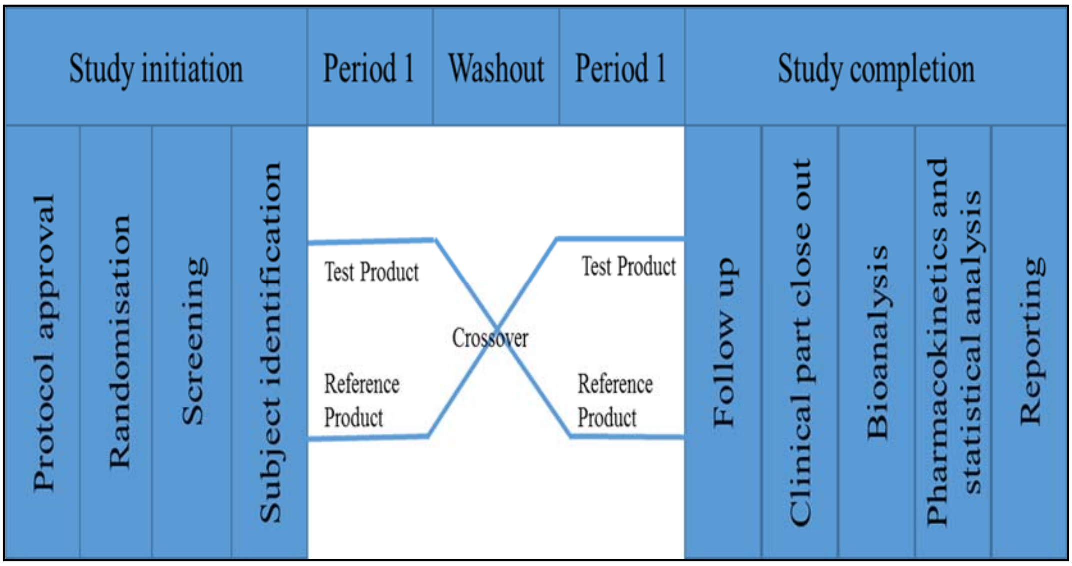

Figure 6.2: Study design and plan

Adequate quantities of the study formulation were provided. A pre-planned scheme was followed as detailed in (Figure 6.2) and the study schematic in (Table 6.5).

Table 6.5: The pre planned scheme, which was conducted for the bioequivalence study

Procedure	**Study period***			
	Screening◆	**Period 1**	**Period 2**	**Follow Up#**
Subject identification	✓	✓	✓	✓
Informed consent♣	✓			
Demographic data	✓			
Medical History	✓			
Physical Examination	✓			✓
Vital signs	✓	✓	✓	
Hepatitis B	✓			
Hepatitis C	✓			
HIV	✓			
ECG	✓			
Haematology	✓			
Biochemistry	✓			✓**
Urinalysis	✓			
Drugs of Abuse		✓	✓	
Alcohol screening		✓	✓	
Selection Criteria°	✓			
Study drug administration		✓	✓	
Check for other medications	✓	✓	✓	✓
Blood Sampling for pharmacokinetics		✓	✓	
Check for adverse events		✓	✓	✓

* There was a washout period of at least 21 days between the two administrations of study drugs

◆Between 14 days and approximately one day before first study drug administration in study period 1

° To be eligible for participation in the study, test subjects must meet all selection criteria before the first study drug administration in study period 1 was established.

♣ Before screening examination, the subject has to sign the informed consent form.

Follow-up was done within at least 24 h of last blood sample

** liver function test

6.6 Adverse events during the study

Test subjects were monitored throughout the confinement period for AE to the study formulation and/or procedures. From the start till the end of the confinement period, a study physician or a medically qualified person were on site and on call. At the beginning of the second period, test subjects were asked concerning unusual symptoms, which may have occurred during the previous administration of the study drug. The qualified medical person or study physician evaluated all drug related symptoms of clinical significance before the next dose administration. Some adverse events occurred during the study but were minimal (Table 6.6). Five out of the twenty-four test subjects experienced AE, with headache, abdominal pain and heartburn being the main adverse effects. All adverse effects occurred during study period 2 and were classified as mild.

Table 6.6: Adverse events that occurred during the bioequivalence study

Subject No.	Adverse event	Study period	Severity	Onset from drug administration (h)	Relationship to study drug	Treatment given	Action taken	Outcome
4	Headache	2	Mild	< 24 h	Possible	A	None	Complete recovery
7	Abdominal pain	2	Mild	< 24 h	Possible	A	None	Complete recovery
8	Heart pain	2	Mild	< 24 h	Possible	A	None	Complete recovery
9	Headache, abdominal pain	2	Mild	< 24 h	Possible	A	None	Complete recovery
14	Abdominal pain	2	Mild	< 24 h	Possible	B	None	Complete recovery

6.7 Dietary restriction

From 48 h prior to the study drug administration, no consumption of alcohol was permitted until the collection of the last sample of the respective study period. Any beverages or foods containing methyl-xanthines such as caffeine (coffee, tea, cola, cocoa, chocolate, etc.) were prohibited for the subjects, 24 h prior to the study drug administration until the end of the confinement in each study period. In addition, any foods or beverages containing grapefruit were prohibited one week before the first study drug administration up until the end of the study. Food and fluid intake were kept identical in both study periods, commencing with dinner served 10 h before study drug administration on study day one until the end of confinement. Test subjects were only allowed to consume foods provided within the period of confinement. All test subjects received meals in the following time frame intervals, as shown in (Table 6.7).

Table 6.7: Standardised meals served during the bioequivalence study

Study day	Standardized diet	Time received
−1	Dinner	Finished at least 10 h before the scheduled time of study drug administration in the morning of study day 1
1	Lunch	4 h after study drug administration
1	Snack	8 h after study drug administration
1	Dinner	12 h after study drug administration

6.8 Blood sample collection and analysis

In the morning of study day 1 of each study period and before study drug administration, a cannula was inserted into the subjects' forearm vein and remained there until the 24 hour blood sample was collected and then the subject returned to donate the rest of samples. The volume of blood taken for determination of azithromycin in plasma was 8 mL per sample. The following blood samples for the analysis of azithromycin in plasma were collected immediately before (2 x 8 mL) at 0.00 (pre-dose) and 0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00 h, (1 x 8 mL) after administration of study drugs (Appendix 5). The number of blood collections for drug analysis was 22 samples in each study period. After centrifugation, plasma samples were transferred directly into a 5 mL tube. These samples were immediately stored at the study site in a freezer at a nominal temperature of −20 °C. The label of collecting tubes had the study's code, subject number, study period and the designated sample number. They did not contain information that would allow identification of the given treatment. This assured that the analysis at IPRC analysed the samples blindly. A validation with LC/MS/MS detector for the determination of azithromycin in human plasma which was developed and validated in accordance with international guidelines at the IPRC was used (ICH Harmonised Tripartite Guideline, 1996, EURACHEM Guide, 1998, Food and Drug Administration, 2001).

6.9 Results

Demographic data and all clinical assessments along with laboratory evaluation were performed for all enrolled subjects. However, for pharmacokinetic evaluations the data from 24 subjects were included in the calculation (Table 6.8 and Table 6.9). Drug plasma levels were designated as surrogate parameters to indicate clinical activity. Primary pharmacokinetic parameters were set to be C_{max} and truncated AUC_{0-72} and were also considered to be the bioequivalence determinants. Finally, t_{max}, $AUC_{0-\infty}$ and $t_{1/2}$ were set as the secondary pharmacokinetic parameters.

The details of azithromycin results for this bioequivalence study are shown in Tables 6.10, 6.11, 6.12, 6.13 and 6.14. Bioequivalence could be demonstrated for azithromycin within the prescribed 90% confidence interval of 80.00-125.00% for C_{max} and truncated AUC_{0-72}.

The test product, Mazit capsules 250 mg (Neopharma, UAE; 250 mg azithromycin per capsule), investigated in this study was shown to be bioequivalent with the reference product Zithromax™ (Pfizer Italia S.R.L., Italy, 250 mg azithromycin per capsule) following an oral dosage of 500 mg (two capsules). Plasma levels may be used as surrogate parameters for clinical activity. Therefore, the data obtained in this study prove, by appropriate statistical methods, the essential similarity of plasma levels of azithromycin from the test product Zithromax™ (Pfizer Italia, S.R.L., Italy) suggesting equal clinical efficacy of these two products.

Table 6.8: Individual plasma concentration of azithromycin versus time after single dose administration of 500 mg azithromycin

Treatment A test product: Mazit capsules 250 mg, 250 mg azithromycin per capsule

	Azithromycin Plasma Concentration (ng/mL)																				
Subject	**Time (Hours)**																				
No.	**0.00**	**0.33**	**0.66**	**1.00**	**1.33**	**1.66**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**16.00**	**24.00**	**48.00**	**72.00**
1	<	53	10	23	19	221	80	141	155	66	62	77	74	62	18	23	29	23	29	<	<
2	<	71	335	651	253	205	250	311	180	130	174	149	121	89	67	37	40	37	25	11	<
3	<	<	31	<	<	<	<	100	234	87	205	140	98	80	47	35	30	20	14	16	12
4	<	<	<	<	<	<	<	<	40	154	102	103	93	81	67	49	50	25	13	***	<
5	<	<	36	43	54	22	18	183	430	317	144	136	118	123	78	62	49	45	32	17	12
6	<	<	71	89	68	72	142	264	219	126	151	197	158	66	57	58	38	34	20	11	<
7	<	30	19	15	106	101	337	161	190	127	128	145	109	77	66	68	54	25	44	13	18
8	<	<	86	78	32	87	17	159	307	113	88	181	124	73	62	66	37	24	26	12	12
9	<	<	12	13	10	12	11	10	20	121	58	37	258	134	62	60	40	30	19	10	<
10	<	<	76	25	40	144	188	221	115	136	102	185	124	89	72	47	44	37	32	***	***
11	<	28	33	33	146	192	287	224	130	130	115	94	68	71	42	31	32	30	24	***	<
12	<	<	40	123	292	337	304	268	281	185	144	179	127	121	64	56	48	42	30	15	14
13	<	11	32	45	76	124	272	223	161	149	227	161	151	120	79	53	47	43	26	19	14
14	<	63	37	16	11	12	200	227	93	95	67	76	59	97	56	38	46	31	25	19	15
15	<	13	11	132	293	242	188	127	176	153	118	156	97	66	62	47	36	24	20	<	<
16	<	<	<	21	28	171	154	96	88	114	135	129	70	69	47	30	28	23	20	<	<
17	<	<	16	23	39	118	97	48	53	37	43	39	56	40	25	15	22	21	18	<	<
18	<	<	20	<	<	<	131	209	131	81	72	79	82	61	41	32	33	31	18	<	<

<: below lower limit of quantification. (LLOQ), were considered zero in the calculation of the statistics.

***: the subject did not give the sample.

Table 6.8 Continued…

	Azithromycin Plasma Concentration (ng/mL)																				
Subject	**Time (Hours)**																				
No.	**0.00**	**0.33**	**0.66**	**1.00**	**1.33**	**1.66**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**16.00**	**24.00**	**48.00**	**72.00**
19	<	<	31	13	79	213	281	126	119	89	71	60	81	81	61	44	34	42	28	14	13
20	<	23	28	19	29	335	371	239	200	158	153	135	204	155	98	69	58	54	43	***	***
21	<	<	63	36	57	178	194	208	126	120	86	81	79	63	45	36	30	39	32	18	12
22	<	<	14	17	19	15	54	133	88	97	97	406	199	123	53	51	45	34	18	19	11
23	<	14	20	91	126	187	156	61	83	246	104	94	76	73	51	37	31	24	20	13	<
24	<	<	19	23	17	56	91	81	103	73	82	73	62	50	39	34	25	26	15	<	<
N	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	20	22
Mean	0	13	43	64	75	127	159	159	155	129	114	130	112	86	57	45	39	32	25	10	6
SD	0.00	21.48	66.19	130.49	88.28	103.08	113.04	83.60	92.50	58.50	46.56	75.24	51.18	28.96	17.44	14.64	9.59	8.95	8.16	7.45	6.91
CV%	#	168.51	152.74	204.82	118.10	81.27	70.97	52.52	59.65	45.23	40.96	58.03	45.69	33.67	30.80	32.59	24.86	28.12	33.12	71.98	114.31
SEM	0.00	4.39	13.51	26.64	18.02	21.04	23.07	17.06	18.88	11.94	9.50	15.36	10.45	5.91	3.56	2.99	1.96	1.83	1.66	1.67	1.47
Minimum	0	11	10	13	10	12	11	10	20	37	43	37	56	40	18	15	22	20	13	10	11
Medium	#	28	31	25	54	144	172	161	131	124	103	132	98	79	59	46	38	31	25	15	13
Maximum	0	71	335	651	293	337	371	311	430	317	227	406	258	155	98	69	58	54	44	19	18

<: below lower limit of quantification. (LLOQ), were considered zero in the calculation of the statistics.

***: the subject did not give the sample.

#: value cannot be calculated

Table 6.9: Individual plasma concentration of azithromycin versus time after single dose administration of 500 mg azithromycin Treatment B reference product: Zithromax™, 250 mg of azithromycin per capsule

	Azithromycin Plasma Concentration (ng/mL)																				
Subject	Time (Hours)																				
No.	0.00	0.33	0.66	1.00	1.33	1.66	2.00	2.50	3.00	3.50	4.00	4.50	5.00	6.00	8.00	10.00	12.00	16.00	24.00	48.00	72.00
1	<	<	<	27	10	64	115	110	94	116	64	50	110	57	38	27	24	21	<	<	<
2	<	<	20	31	87	203	239	279	210	126	138	123	104	66	40	40	27	18	13	<	<
3	<	<	15	<	<	<	<	<	173	384	246	174	106	106	75	65	47	34	14	11	<
4	<	10	87	25	14	72	37	72	219	257	138	106	104	126	77	64	47	24	26	<	***
5	<	<	41	34	50	127	316	389	239	122	111	134	134	95	64	49	34	38	33	15	12
6	<	<	55	48	130	255	194	121	113	114	96	106	86	60	43	37	30	19	17	10	<
7	<	<	17	17	195	424	186	222	178	220	58	172	114	118	66	71	53	19	36	12	27
8	<	12	112	38	106	329	265	308	163	101	95	96	80	59	46	59	27	22	27	11	14
9	<	<	37	42	27	18	15	140	135	159	92	157	162	140	74	60	47	31	25	***	10
10	<	12	168	88	196	237	199	109	94	113	95	105	91	78	46	44	41	41	29	19	14
11	<	16	11	<	<	77	286	290	126	122	120	104	91	67	42	35	39	33	20	***	<
12	<	<	37	16	21	322	528	387	294	307	187	181	114	105	97	72	49	44	31	18	13
13	<	<	29	22	26	27	235	272	106	147	167	147	114	78	84	55	40	36	27	20	15
14	<	<	59	22	11	10	12	182	254	196	182	199	146	112	83	73	61	48	29	22	20
15	<	40	15	165	346	295	406	158	190	206	112	95	88	49	43	46	43	38	25	11	<
16	<	<	<	33	27	18	14	129	150	129	46	122	111	59	46	39	37	24	19	<	<
17	<	<	<	<	11	142	156	76	70	68	62	86	78	106	39	23	31	23	19	13	<
18	<	15	22	11	14	124	162	128	88	68	93	75	92	50	30	31	21	18	18	16	<

<: below lower limit of quantification. (LLOQ), were considered zero in the calculation of the statistics.

***: the subject did not give the sample.

Table 6.9 continued…

Azithromycin Plasma Concentration (ng/mL)																					
Subject	**Time (Hours)**																				
No.	**0.00**	**0.33**	**0.66**	**1.00**	**1.33**	**1.66**	**2.00**	**2.50**	**3.00**	**3.50**	**4.00**	**4.50**	**5.00**	**6.00**	**8.00**	**10.00**	**12.00**	**16.00**	**24.00**	**48.00**	**72.00**
19	<	<	12	<	24	272	434	189	135	*	150	146	177	223	115	88	58	58	33	***	***
20	<	<	38	11	<	15	177	407	222	153	145	151	156	95	101	87	72	72	50	21	18
21	<	23	53	169	125	203	325	174	154	148	91	101	100	63	45	33	31	24	27	22	10
22	<	<	29	42	28	14	35	62	122	150	230	185	173	131	68	46	50	38	25	12	<
23	<	<	<	13	14	11	16	168	48	57	167	99	57	58	30	34	29	23	11	<	<
24	<	<	14	20	26	88	238	142	135	71	52	75	60	66	36	32	29	21	18	11	<
N	24	24	24	24	24	24	24	24	24	23	24	24	24	24	24	24	24	24	24	21	22
Mean	0	5	36	36	62	139	191	188	155	154	122	125	110	90	60	50	40	32	24	12	7
SD	0.00	10.03	39.38	44.65	84.25	126.18	146.11	109.61	61.80	79.19	54.19	39.69	32.79	39.64	24.15	18.72	12.85	13.69	9.93	7.68	8.50
CV%	#	188.12	108.51	122.62	135.89	90.48	76.40	58.28	39.96	51.54	44.28	31.87	29.72	43.90	40.59	37.13	31.90	42.85	41.66	66.13	122.29
SEM	0.00	2.05	8.04	9.11	17.20	25.76	29.82	22.37	12.62	16.51	11.06	8.10	6.69	8.09	4.93	3.82	2.62	2.80	2.03	1.68	1.81
Minimum	0	10	11	11	10	10	12	62	48	57	46	50	57	49	30	23	21	18	11	10	10
Medium	#	15	33	29	27	124	194	168	143	129	112	114	105	78	46	46	40	28	25	14	14
Maximum	0	40	168	169	346	424	528	407	294	384	246	199	177	223	115	88	72	72	50	22	27

<: below lower limit of quantification. (LLOQ), were considered zero in the calculation of the statistics.

***: the subject did not give the sample.

#: value cannot be calculated

*: broken in clinical site

Table 6.10: Individual pharmacokinetics of azithromycin after single dose administration of 500 mg azithromycin Treatment A test product: Mazit capsules 250 mg, 250 mg of azithromycin per capsule

Subject No.	C_{max} (ng/mL)	T_{max} (h)	AUC $_{0-72}$ (ng.h/mL)	AUC $_{0-\infty}$ (ng.h/mL)	$t_{1/2}$ (h)
1	221	2.00	990.5	---	---
2	651	1.00	2389.2	2708.2	20.10
3	234	3.00	1712.9	---	---
4	154	3.50	974.8	1100.6	6.71
5	430	3.00	2698.2	3196.9	28.80
6	264	2.50	1855.8	2175.1	20.12
7	337	2.00	2651.8	3405.6	29.03
8	307	3.00	2087.3	2751.5	38.37
9	258	5.00	1505.8	1812.8	21.27
10	221	2.50	1500.2	2666.2	25.26
11	287	2.00	1286.4	2264.7	28.25
12	337	1.66	2840.8	3448.3	30.08
13	272	2.00	2667.00	3752.6	53.75
14	227	2.50	2146.9	3556.4	65.13
15	293	1.33	1395.7	1674.5	9.66
16	171	1.66	1031.2	1720.3	23.88
17	118	1.66	662.2	1715.7	40.57
18	209	2.50	1003.7	1343.3	13.08
19	281	2.00	2148.9	2779.2	33.60
20	371	2.00	2133.00	3823.1	27.24
21	208	2.50	2242.6	2828.7	33.85
22	406	4.50	2211.6	2686.2	29.90
23	246	3.50	1543.4	2206.9	35.38
24	103	3.00	841.2	1110.00	12.42
N	24	24	24	22	22
Mean	275	2.51	1771.7	2487.6	28.48
SD	114.32	0.94	657.78	840.75	13.60
CV%	41.53	37.55	37.13	33.80	47.75
SEM	23.34	0.19	134.27	179.25	2.90
Median	261	2.5	1784.35	2676.2	28.53
Min	103	1.00	662.2	1100.6	6.71
Max	651	5.00	2840.8	3823.1	65.13

---No clear elimination

Table 6.11: Individual pharmacokinetics of azithromycin after single dose administration of 500 mg azithromycin treatment B reference product: Zithromax™, 250 mg of azithromycin per capsule

Subject No.	C_{max} (ng/mL)	T_{max} (h)	AUC $_{0-72}$ (ng.h/mL)	AUC $_{0-\infty}$ (ng.h/mL)	$t_{1/2}$ (h)
1	116	4	700.1	1216.4	17.04
2	279	2.5	1230.2	1402.8	9.2
3	384	3.5	1713	1921.8	13.16
4	257	3.5	1419.1	1701.4	7.53
5	389	2.5	2545.4	3093.1	31.64
6	255	1.66	1429.8	1916.9	33.76
7	424	2	2807.6	3213.5	31.71
8	329	1.66	2127.7	2762.7	31.44
9	162	5	2255.7	2760.4	34.98
10	237	1.66	2397.6	3320.4	45.69
11	290	2.5	1253.6	1606.7	12.24
12	528	2	3092.4	3793.8	37.4
13	272	2.5	2441.6	3637.8	55.27
14	254	3	2815.7	5332.9	87.24
15	406	2	2044	2332.6	18.18
16	150	3	992.6	1298.2	11.15
17	156	2	1330.2	2078.9	39.92
18	162	2	1273.2	5309.7	174.87
19	434	2	2193.8	2729.4	11.25
20	407	2.5	3438.3	4081.5	24.77
21	325	2	2304.2	2787.5	33.5
22	230	4	1903.2	2248.3	19.94
23	168	2.5	815.6	949.1	8.41
24	238	2	1299.9	1842.3	34.18
N	24	24	24	24	24
Mean	286	2.58	1909.4	2639.1	34.35
SD	108.75	0.86	743.82	1184.01	34.93
CV%	38.09	33.49	38.96	44.86	101.69
SEM	22.20	0.18	151.83	241.69	7.13
Median	265	2.50	1973.6	2531	31.54
Min	116	1.66	700.1	949.1	7.53
Max	528	5.00	3438.3	5332.9	174.87

Table 6.12: Ratio analysis of untransformed C_{max} data of azithromycin after an oral dose administration of 500 mg azithromycin of Treatment A test product Mazit capsules, 250 mg of azithromycin per capsule and Treatment B reference product Zithromax™, 250 mg of azithromycin per capsule

C_{max}			
Subject No.	**TEST**	**REFERENCE**	**TEST/REFERENCE**
	Data	**Data**	**Data**
1	221	116	190.52
2	651	279	233.33
3	234	384	60.94
4	154	257	59.92
5	430	389	110.54
6	264	255	103.53
7	337	424	79.48
8	307	329	93.31
9	258	162	159.26
10	221	237	93.25
11	287	290	98.97
12	337	528	63.83
13	272	272	100.00
14	227	254	89.37
15	293	406	72.17
16	171	150	114.00
17	118	156	75.64
18	209	162	129.01
19	281	434	64.75
20	371	407	91.15
21	208	325	64.00
22	406	230	176.52
23	246	168	146.43
24	103	238	43.28
N	24	24	24
Mean	275	286	104.72
GM	255	265	96.17
SD	114.32	108.75	46.81
CV%	41.53	38.09	44.70
SEM	23.34	22.20	9.55
90% CI of Geometric Means	Point estimate %		96.16
	Lower limit %		82.95
	Upper limit %		111.51

Table 6.13: Ratio analysis of untransformed AUC_{0-72} data of azithromycin after an oral dose administration of 500 mg azithromycin of Treatment A test product Mazit capsules, 250 mg of azithromycin per capsule and Treatment B reference product Zithromax™, 250 mg of azithromycin per capsule

AUC_{0-72}			
Subject No.	**TEST**	**REFERENCE**	**TEST/REFERENCE**
	Data	**Data**	**Data**
1	990.5	700.1	141.48
2	2389.2	1230.2	194.21
3	1712.9	1713	99.99
4	974.8	1419.1	68.69
5	2698.2	2545.4	106.00
6	1855.8	1429.8	129.79
7	2651.8	2807.6	94.45
8	2087.3	2127.7	98.10
9	1505.8	2255.7	66.76
10	1500.2	2397.6	62.57
11	1286.4	1253.6	102.62
12	2840.8	3092.4	91.86
13	2667.00	2441.6	109.23
14	2146.9	2815.7	76.25
15	1395.7	2044	68.28
16	1031.2	992.6	103.89
17	662.2	1330.2	49.78
18	1003.7	1273.2	78.83
19	2148.9	2193.8	97.95
20	2133.00	3438.3	62.04
21	2242.6	2304.2	97.33
22	2211.6	1903.2	116.20
23	1543.4	815.6	189.23
24	841.2	1299.9	64.71
N	24	24	24
Mean	1771.7	1909.4	98.76
GM	1641	1761	93.21
SD	657.78	743.82	36.50
CV%	37.13	38.96	36.96
SEM	134.27	151.83	7.45
90% CI of Geometric Means	Point estimate %		93.22
	Lower limit %		82.64
	Upper limit %		105.12

Table 6.14: Ratio analysis of untransformed $AUC_{0-\infty}$ data of azithromycin after an oral dose administration of 500 mg azithromycin of Treatment A test product Mazit capsules, 250 mg of azithromycin per capsule and Treatment B reference product Zithromax™, 250 mg of azithromycin per capsule

$AUC_{0-\infty}$			
Subject No.	**TEST**	**REFERENCE**	**TEST/REFERENCE**
	Data	**Data**	**Data**
1	---	1216.4	0.00
2	2708.2	1402.8	193.06
3	---	1921.8	0.00
4	1100.6	1701.4	64.69
5	3196.9	3093.1	103.36
6	2175.1	1916.9	113.47
7	3405.6	3213.5	105.98
8	2751.5	2762.7	99.59
9	1812.8	2760.4	65.67
10	2666.2	3320.4	80.30
11	2264.7	1606.7	140.95
12	3448.3	3793.8	90.89
13	3752.6	3637.8	103.16
14	3556.4	5332.9	66.69
15	1674.5	2332.6	71.79
16	1720.3	1298.2	132.51
17	1715.7	2078.9	82.53
18	1343.3	5309.7	25.30
19	2779.2	2729.4	101.82
20	3823.1	4081.5	93.67
21	2828.7	2787.5	101.48
22	2686.2	2248.3	119.48
23	2206.9	949.1	232.53
24	1110.00	1842.3	60.25
N	22	24	22
Mean	2487.6	2639.1	102
GM	2337	2397	93.56
SD	840.75	1184.01	44.60
CV%	33.80	44.86	43.63
SEM	179.25	241.69	9.51
90% CI of Geometric Means	Point estimate %		93.56
	Lower limit %		80.06
	Upper limit %		109.32

---No clear elimination

6.9.1 The concentration of Mazit versus Zithromax for all subjects

The concentration and logarithmic concentration of the test product, Mazit capsules (Neopharma, UAE; 250 mg azithromycin per capsule), and the reference product Zithromax™ (Pfizer Italia S.R.L., Italy, 250 mg azithromycin per capsule) after an oral dosage of 500 mg (two capsules) in subject 1 is shown in the figures below.

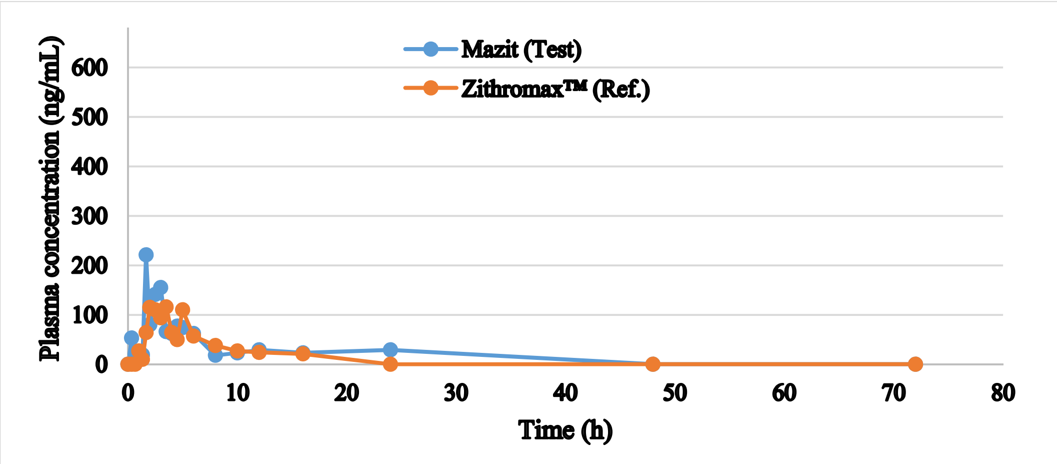

Figure 6.3: Concentration of Mazit compared to Zithromax™ (Subject 1)

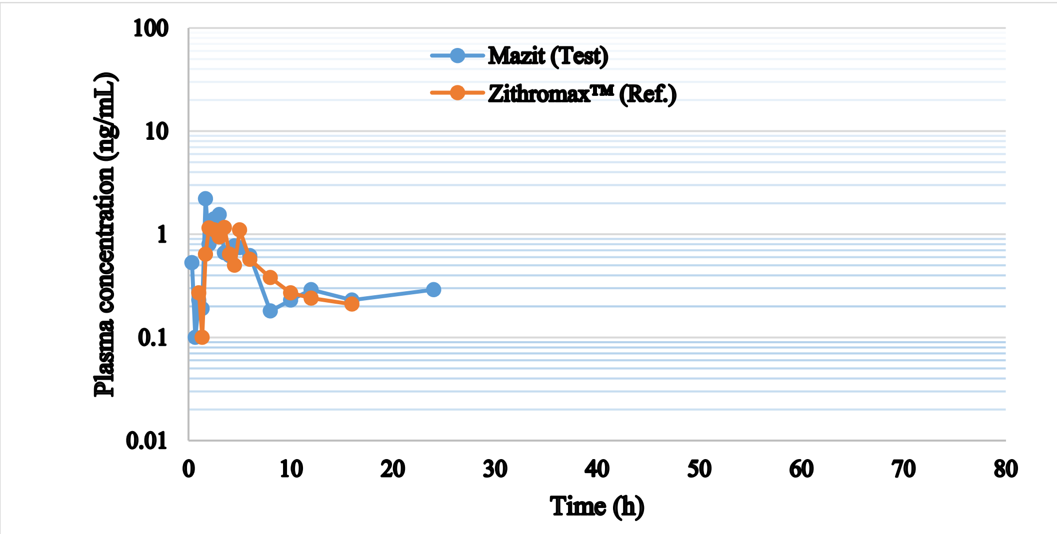

Figure 6.4: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 1)

Figures for subjects 2-24 are in appendix 6

6.10 Conclusions

This study was a single centre, open label, randomised, single dose study with two-way crossover design to compare the bioavailability of azithromycin between two products, in healthy, adult volunteers under fasting conditions. The results of this bioequivalence study showed the equivalence of the two studied products in terms of the rate of absorption as indicated by C_{max} and in terms of the extent of absorption as indicated by AUC_{0-72} and $AUC_{0-\infty}$. The parametric 90% confidence intervals of the mean values for the test/reference ratio were in each case well within the bioequivalence acceptable boundaries of 80-125% for C_{max} and truncated AUC_{0-72} (Appendix 8).

Since plasma levels are a meaningful surrogate for pharmacodynamic action and adverse events, this demonstrates that an equivalent therapeutic activity and tolerance is to be expected from Mazit capsules 250 mg (Neopharma, UAE) test product as compared to Zithromax™ (Pfizer Italia, S.R.L., Italy), the reference product.

Chapter 7. General discussion and conclusion

Based on current growth trends, by the time this thesis is finished (2015), it is estimated that the counterfeit market will be worth over $150 billion a year. In 2000, there was a WHO report on Africa and Asia, confirming that between 38% and 53% of antimalarial drugs were counterfeit, with no active ingredients in the drug product. An estimated 700,000 deaths yearly are from substandard antimalarial and anti-TB medications (Mackey and Liang, 2011). During 2003 the estimated yearly profits made from substandard and counterfeits were more than $32 billion (World Health Organization, 2003). As mentioned in chapter 1, as of 2010, this estimate has more than doubled. This is evidence of the ever-increasing challenges faced by regulatory bodies and why it is such a lucrative industry. Counterfeiters find vulnerabilities in the system, as the incentives to abuse the healthcare industry, as mentioned previously, are very high. Authorities and regulators are moving far too slowly in terms of reacting to vulnerabilities in the current system.

One of the points of entry for counterfeit or substandard medicines is the high percentage of active ingredient's now imported into the United States, from regions where regulations and quality control are not as rigorous (Blackstone et al., 2014). Due to some vulnerabilities in the manufacturing and supply chains, counterfeit and substandard manufacturers can make their way through the European supply chains via countries such as China, India, Pakistan, Spain and Russia, that have low regulation and quality controls, as most European countries import their medications from these regions (Bate, 2008). Further regulation and monitoring processes should be applied to poorly regulated areas. It is extremely difficult to prevent counterfeit drugs entering a country such as United States as nearly 40% of drugs are manufactured overseas and approximately 80% of the active ingredients used for all pharmaceuticals are imported into the United States (Blackstone et al., 2014).

In this thesis, approaches to the investigation of counterfeit drugs have been studied and a number of example drugs fully investigated using the described techniques. The *in-vitro* dissolution testing was found to be able to identify differences in dissolution performance between branded and generic products. Dissolution testing was carried out according to the United States Pharmacopeia (USP) guidelines. According to the USP, not less than 80% of the labelled amount of ciclosporin and azithromycin should dissolve in 90 and 30 min, respectively. The samples were analysed using liquid chromatography.

Dissolution testing was useful in determining the difference in dissolution timings of different tablets and capsules. In addition, it was useful as a preparatory technique in order to prepare samples for further analysis (e.g.: drug analysis using LC or CE). Therefore, the dissolution tester should be available in every drug analysis laboratory.

In this study, the LC systems with simple UV detection were used to determine the drug content of capsules and tablets. In addition, the LC system was also found to be useful in determining the presence of impurities in ciclosporin capsules. However, to determine the nature and the relative quantities of these impurities required more selective detection, so a LC-MS is necessary. Thus, it is recommended to use a combination of LC-UV and LC-MS for identifying content and impurities, respectively.

In contrast, the LC-MS system used for identifying impurities was unable to provide absolute quantities of the impurities. In order to derive absolute quantities using LC-MS, a set of external calibrator and labelled internal standards are required. For most of the impurities, there were no available external calibrator/internal standards. Therefore, it was difficult to perform absolute quantification. However, further studies are required to perform such quantitation.

7.1 Dissolution testing

Dissolution testing can be used to test the overall drug formulation if there is suspicion of it being counterfeit or substandard. A dissolution method is suitable for testing drugs to monitor the effective release of the active ingredients. The guidelines for dissolution testing can be obtained from sources such as the USP and FDA. Dissolution test could be used during the formulation design stage of the drug. It is an important measurement for quality control of a drug. This gives an indication of the timing of which the drug is released (*in-vivo*) after consumption and could highlight any potential differences and bioavailability. The medium used in ciclosporin dissolution test was only water, while in azithromycin dissolution test it was aqueous with 0.1 M phosphate buffer (pH 6.0), which met the USP and FDA guidelines for dissolution testing. It is safe to consider that the choice of water as an appropriate bio-relevant dissolution medium as its used for quality control testing (Qureshi, 2010). Although, further review for the dissolution testing guidelines should be considered to make the dissolution testing closer to the GI environment, especially important for ionisable drugs. A study of an *in-vitro* dissolution test on amoxicillin concluded that 62.5% of the tested generics were not substitutable for the brand Amoxil™ (Kassaye and Genete, 2013).

7.2 Ciclosporin study

Since ciclosporin is an important immunosuppressant agent, it requires careful therapeutic dosing. In recent years, many studies have questioned the use of generic substitutes, especially when considering those that are NTIDs.

In this test the aim was to develop, validate and apply a dissolution test for ciclosporin in capsules using a HPLC method and then to compare brand medications to the generic or copy products. Moreover, the detection of contaminates and the source of impurities in these products are essential to understand.

A sensitive and specific HPLC-UV method was developed to detect and subsequently quantitate the ciclosporin active base in capsules. Dissolution testing of the capsules was done as specified in the USP. Analytes were isocratically eluted at 0.7 mL/min with acetonitrile and water (70:30% v/v) and 0.03% v/v trifluroacetic acid over the 25 min runtime. Although the USP recommends that the column should be heated to 80°C, there is a difference in the temperature researchers choose that fits with their respective system. When this method was applied, it did not achieve the required separation, but at 50°C the necessary sensitivity and separation was achieved. The HPLC method developed in this study is significantly different to that stated in the USP.

Treating patients orally, using capsules or tablets is still one of the most used routes of administration. After oral administration of capsules, the capsule shell should rupture within a specific time to allow the release of the drug in the gastrointestinal tract before going to the systemic circulation. The rate of dissolution of the capsule is therefore essential. Dissolution profiles revealed that all the ciclosporin capsules used in these investigations complied with USP, rupturing within 15 min (The United States Pharmacopeia, 2008). There are discrepancies in the dissolution methods between the FDA and the USP. The FDA suggest to use a litre of 0.1 N HCl containing 4 mg of N,N-dimethydodecylamine-N-oxide per mL at 75 rpm. The USP in 2012 recommend the use of 500 mL water at 50 rpm (Food and Drug Administration, 2008a, USP 35, 2012).

According to the USP, the guideline for dissolution sampling is at 90 min for ciclosporin which requires a minimum of 80% of the active ingredient. As part of the study reported here, additional sampling times up to 2 h were required to monitor the dissolution behaviours.

The standard curve, when fitted using least square linear regression, was linear. The LC successfully separate the compound of interest. Detection of ciclosporin at low and high concentration was reproducible and the method was sensitive. The method was specific, as the blank runs did not produce any interfering peaks coinciding with retention time of ciclosporin. The within day and between day variability of the standards was within an acceptable range. The method was accurate. There was no system carry over for ciclosporin. Blank samples were run between the actual samples without detection of any carry-over of ciclosporin.

The application of the assay was successful. The Turkish brand was the best brand in terms of content of the active ingredient with 100 ± 0.05% and 100 ± 0.03% for both test conditions. Generics from India and Morocco had only 69 ± 0.08% and 54 ± 0.10 %, respectively. There were one or more impurities in all capsules.

It was confirmed in this thesis, that there is a difference in dissolution behaviours between generic ciclosporin and its branded counterpart. In Tables 4.3 and 4.11, ciclosporin test A and B, there was a noticeable early release of ciclosporin after 5 min sampling time of generic C and generic Col. The average percentage content of ciclosporin found, was 85% and 81%, respectively. During this sampling time, none of the other tested brands and generics had that much release of ciclosporin. The reason for the rapid ciclosporin release for the above mentioned generic could be due to the thin transparent capsule shell. These ciclosporin capsules met the USP requirements, rupturing within 15 min but there is no clear information about the effects of early release of ciclosporin. It was found in test A, that generic I average content was less than the required USP amount (69.3% after 90 min). Moreover, in test B, the average content of ciclosporin M, was found to be 54% only after 90 min. This indicates that these two products could be substandard and/or counterfeit. The contents of one of the ciclosporin capsules we reported in this study is in agreement with those found by other researchers (Bonifacio et al., 2009), where they detected only 68% of the labelled concentration in one of the generic capsules. Using such products with lower therapeutic doses in transplant patients could lead to acute rejection (Browne et al., 2001). Even within brands of ciclosporin from different countries, there were differences in the average content after 90 min within the acceptable limit. In Table 4.11, at 90 min sampling times, the average content of ciclosporin for TK, Sa, Jor, Egy and Pak was 100, 99, 84, 92, and 94%, respectively. This indicates that even within the same brand, there are differences in the average content of ciclosporin. Some small differences in NTIDs such as ciclosporin, could lead to harmful side effects. Based on these results, switching between generic and branded ciclosporin is not recommended. The tests provided some surprising results. Some of the test products had less than 80% requirement and even some of the branded drugs which are supposed to have set the benchmark did not have 100% either. This highlights potential poor standards in the manufacturing process which need to be reviewed.

While using the HPLC method to quantify ciclosporin labelled amount, some impurities were detected using the UV detector (Figure 4.2). The impurities in ciclosporin capsules were then investigated using a UHPLC-MS in order to identify the nature of these impurities. Samples were then analysed for the whole chemical content using a Q-TOF LC-MS system and compared to each other using univariate (fold change and p values) and multivariate (PCA and heatmaps) statistical methods. All generic capsules were found to have a different chemical content when compared to the brand product. On identifying the nature of impurities in one of the generic compounds (Col), sorbitol was found to be higher by 260 fold change. Sorbitol is widely used in the pharmaceutical industry as an inactive ingredient, its function being to sweeten medicines such as syrups and chewable tablets. It is synthesised by hydrogenation of glucose and is formulated into a liquid or crystalline preparation. Sorbitol is less sweet than glucose and contains less calories. In ciclosporin capsules, sorbitol is used as a plasticizer (Rowe et al., 2009).

A known side effect of sorbitol is its laxative effect but as it is administered in small quantities in pharmaceuticals, this is not a common occurrence.

Other side effects may include anal irritation, diarrhoea, gas, stomach cramps, and nausea. It also has potential serious side effects which require medical intervention such as allergic reactions concerning rash, itching, difficulty breathing, swelling of the mouth, face, lips or tongue and rectal bleeding (UpToDate, 2015b).

According to the FDA, the maximum amount of sorbitol in soft gelatine liquid filled capsules (such as ciclosporin) should be 97 mg (Food and Drug Administration, 2015c). However, there is no listed label quantity of sorbitol as an inactive ingredient in ciclosporin capsule products. Even though the amount of sorbitol as an inactive ingredient is not listed in the ciclosporin product, a high amount of sorbitol can cause some serious side effects (Rowe et al., 2009).

Other contaminants such as the ciclosporin degradation product delcorine and the plant contaminant zizyphine A, were also found in the generic product. Their safety and immunosuppressive activity are unknown and need further investigation.

The results in this study were surprising in that the content was much less than 80% in some of the capsules and was not 100 % in all the brand capsules either. This raises concerns over the manufacturing standards and the quality control measures employed even within the same brand manufactured in different countries. In the literature, the Danish medicine agencies suggested that generic substitution should no longer be used for ciclosporin following a reassessment (Heisterberg, 2011). Taking together, this data highlights the need for more quality control test of generic products and post marketing pharmacovigilance.

7.3 Azithromycin study

Azithromycin is a widely used antibiotic. In India alone, there are 40 manufacturers producing 54 generic versions (Medindia, 2015). It was selected for this study due to an FDA warning about the potential fatality risk. According to Kelesidis and Falagas, as of 2009, 11% of counterfeit antimicrobial medications worldwide, were macrolides (Kelesidis and Falagas, 2015). The purpose of this study was to quantify the percentage labelled amount of azithromycin in brand versus generic copies, after *in-vitro* dissolution testing. According to the USP not less than 80% of the labelled amount of azithromycin should dissolve in 30 min.

In this study, azithromycin tablets from different generic preparations were subjected to dissolution testing at six sampling times in order to get results that are more adequate. In the USP monograph about azithromycin tablets, it mentions that the sampling times for dissolution should be done at 30 min, while the FDA provide more sampling times at 10, 20, 30 and 45 min. Five of the generic products studied failed to meet the USP requirements at azithromycin dissolution time of 30 min. On the other hand, after 60 min, only three generic products were below the acceptable limit. The results showed that, some generic tablets were not fully dissolved after 30 min and needed more time to fully dissolve. This indicates a formulation or manufacturing liability with azithromycin tablets.

In this study, a UHPLC-MS/MS method was modified to quantify azithromycin labelled amount in the generic and brand counterpart tablets (Rossmann et al., 2014). Roxithromycin was selected as an IS because it has a comparable structure, retention time and ionisation to azithromycin (Xu et al., 2008), and the method was validated. In this study, the UHPLC-MS/MS azithromycin method was found to be highly sensitive and accurate with acceptable precision. In order to get good peak resolution, previous studies suggested using either ion-pairing reagents or derivatization of azithromycin (Sharma and Mullangi, 2013). The use of ion-pairing agents is associated with unwanted effects such as ion suppression, high background noise and shortening of the LC column life. The use of derivatization agents is also undesirable as they can be toxic. In this study, azithromycin and its internal standard roxithromycin were adequately separated and achieved excellent peak resolution with minimal peak tailing using LC-MS/MS and without using any ion-pairing agents or derivatization method. Furthermore, the run time was short allowing a high sample throughput.

After validation, the method was applied for the measurement of the actual concentration in each azithromycin products. Five generics (D, E, F, H, N) had less than the minimum percentage of labelled amount, indicating a presence of substandard and/or counterfeit medications for consumers. In this study, only three tablets from each product were available for analysis. Results from this study showed that the CV of the analysis of these three tablets was less than 15% for ten products, indicating good reproducibility between analysed tablets for these products. However, another eight products showed CV higher than 15% indicating bad reproducibility and high variability between tablets. Two products showed a CV higher than 50% indicating a serious problem in these tablets. These results would raise concerns about using low quality antibiotics.

With an increase in the estimated sales of counterfeit medicines, quality control tests of antibiotics are essential to ensure effective treatment (Le Doare et al., 2015). Using poor quality antibiotics could result in an increase of bacterial resistance, failure of the treatment and/or toxicity (Kelesidis et al., 2007, Johnston and Holt, 2014, Kelesidis and Falagas, 2015).

7.4 Bioequivalence testing: brand vs generic

Over the last 30 years, bioequivalence testing has become an even more integral process in the pharmaceutical industry. The FDA has implemented many rules and regulations when it comes to bioequivalence testing, from the 1970s until today (Buehler, 2010). Bioequivalence testing is necessary in approving generic drugs. They are an essential benchmark that drugs need to pass in order to confirm they offer the same properties as the branded counterpart. For a generic drug to be approved, the bioavailability should be between 80-125% compared to the branded product (European Medicine Agency, 2001).

In this thesis, a single dose bioequivalence study between Mazit 250 mg and Zithromax™ 250 mg showed that they were bioequivalent within the acceptable limits 80-125% with no significant safety profile observed. Some bioequivalence studies were done on azithromycin and the same conclusion was reached, that the generic was bioequivalent to the branded counterpart (Ren et al., 2007).

In theory, generic drugs can be substituted with their branded counterpart. The fact that the cost of branded medications is higher than generics, encourages insurance companies, pharmacies, doctors and health care organisations to favour generics as an alternative (Silverman, 2015). A Medline search by Meridith, of the data published between 1973-2003 suggested that generic drug substitution should be approached with caution. Bioequivalence tests are done as a single dose study, while multiple doses may be required to reach steady state (Meredith, 2003). Another Medline search from 1966-2006 by Al-Jazairi et al., in 2008, suggested that generic drug substitution is acceptable as long as generics have met the bioavailability limits. However, extra caution and monitoring should be considered with regards to NTIDs (e.g. ciclosporin, warfarin, carbamezapine, thyroxine) and highly variable drugs (e.g. verapamil), where interchangeability is not recommended (Al-Jazairi et al., 2008). A PubMed search from 1974-2010, by Desmarais et al., concluded that the use of generic substitution may lead to some side effects and/or toxicity resulting in more complications and may eliminate cost savings (Desmarais et al., 2011). A systematic review was carried out by Goth et al., in 2015 using Medline, Embase and the Cochrane Database, on generic substitution based on the effectiveness and cost efficiencies compared to its branded counterpart. It suggested that more research is required for generic interchangeability, especially for antiepileptic and immunosuppressive drugs (Gothe et al., 2015). There should be more communication and education about using generic substitutions (Heikkila et al., 2007, Keenum et al., 2012).

However, there is a lot of debate regarding what acceptance limits are required for generic drugs and this is even more important for NTIDs as the slightest variation could have serious effects. Bioequivalence tests for some generic drugs have confirmed that there are bioequivalence variations between a generic and its branded counterpart (Del Tacca et al., 2009). Other opinions in the pharmaceutical industry say that the current generic drug approval system is sufficient to conform to a branded drugs therapeutic equivalence (Motola and De Ponti, 2006).

The use of NTIDs requires a more detailed patient monitoring process, as small differences in bioavailability can have serious effects. The EMA set out new guidelines concerning NTIDs specifically highlighting the acceptance limit of AUC to a tighter range of between 90-111.11% but there is currently no available list confirming all the NTIDs that require this new guideline (European Medicine Agency, 2010). The MHRA announced that ciclosporin is classified as an NTID and warns that patients should be administered the same brand of ciclosporin and if this is not possible to undergo close monitoring (Medicines and Healthcare products Regulatory Agency, 2009). The fact that health regulation authorities have tightened the acceptance range for NTIDs, confirms that the current bioequivalence limit is too wide. This could indicate that, interchanging between brand and generics may lead to unwanted effects.

On the other hand, many systematic reviews and studies suggest that there are no significant differences when switching from brand to generic (Kesselheim et al., 2008, Moore et al., 2009).

The results in this thesis confirm that there are some differences between ciclosporin and azithromycin products, brand versus generic and brand versus brand. This highlights the importance of not using ciclosporin as an interchangeable drug.

7.5 Future control of generic and counterfeit drugs

It is advisable that the health care authorities review the guidelines for approving generic medications on all levels. Post marketing pharmacovigilance is essential to ensure good quality medications are available for patients. Cost savings from using generics may not be real if serious side effects and/or toxicity occur. In a nutshell, it is not about brand or generic but quality and safety.

Several recent conferences have addressed the noticeable increase of concern within the health regulators and pharmaceutical industry about fake medicines.

One suggested solution to decrease the spread of counterfeit and substandard medicines is to review pricing. Price factors are one, if not the main reason for consumers to search for cheaper alternatives. This may incentivise consumers to search for and purchase drugs from online pharmacies, which as mentioned earlier in this thesis, have a much higher chance of being counterfeit or substandard. One solution could be to look at the manufacturing costs that are imposed on pharmaceuticals such as registration costs (Medicines and Healthcare products Regulatory Agency, 2014). If costs associated by regulators are reviewed and reduced, this could result in a reduction in consumers looking for alternatives and lowering chances of potential adverse effects. In addition, many consumers are unaware of the risks from purchasing drugs online from Internet based pharmacies. Further information and warnings are needed to inform consumers of the possible dangers and how to proactively check the authenticity of vendors and of the drugs purchased.

As mentioned in the thesis, there are many stages where counterfeits or substandard production methods can enter or occur. This could be reduced by refining the stages from manufacturing to distribution and by implementing a regular track and trace system where you can follow the whole process of a drug, from manufacturing, to distribution and dispensing can be followed. Manufacturers may not want to disclose who their suppliers are or their business practices, but transparency in the pharmaceutical industry should be essential to lower the risk to public health, reduce funding terrorism, better economic prosperity and reduce the vulnerabilities of counterfeit and substandard drugs entering the supply chain. Pharmaceutical counterfeiting could be the most important criminal activity worldwide, as this funds global terrorism and is a great risk to public safety.

Another solution proposed by the American House Energy & Commerce Sub-committee on Oversight and Investigations hearing on counterfeit drugs was to increase state licensure supervision of drug wholesalers. In today's age of technology, there are options that can be put in place to help fight the issue of counterfeit drugs entering the supply chain. One technology being considered is Radio Frequency Identification (RFID) (Food and Drug Administration, 2005). RFID is a chip that stores a serial number and can confirm the products identity. It can store much more information than a traditional barcode and can add a level of automation and inventory checks that traditional barcodes cannot. RFID has already made its way into adding an additional layer of security in passports. This highlights their reputation as a reliable form of tracking and data storage. An RFID consists of three main components, 1) the transponder, which is the actual radio frequency tag. This is the piece that holds all the information, 2) the antenna, which is the coil that allows information to be read off the RFID tag and 3) the receiver, which is the scanner that decodes the information stored on the RFID tag. RFID tags are available in many sizes. Small RFID tags can be successfully added to individual drug packaging behind labels, which are scanned individually in close range and larger RFID tags to monitor and track large quantities of drugs in batches, as they move within a factory or logistically. In addition, security alerts can be implemented to logistics to monitor where shipments are and automatically notify vulnerabilities like shipments not being recorded as reaching their destination or not arriving at the desired destination within their expected time frame, highlighting possible delays due to counterfeit manipulation or tampering. Another advantage of RFID technology is that it is continuously being improved and innovated, thus allowing the ability to expand and update without the need to invest in a whole new infrastructure from the ground up. Large pharmaceutical companies have already started to implement this technology, AstraZeneca being one of the first. We can agree that the costs to implement and improve security and regulations are high but it has to be focused as a long-term goal. The benefits in reducing counterfeiting and substandard drugs will be felt across many sections and will thus free up revenues that would be otherwise used to react to the many repercussions. A global effort in implementation should be considered as cross border regulations would help reduce counterfeit drugs on a mass scale.

In this thesis, some of the main vulnerabilities in the current pharmaceutical industries and ways in which we can approach to tackle the issues were discussed. The key ones being that the biggest flaws are in the legitimate supply chains. Upon further review, some ideas on cutting down on the levels of the supply chain and the advantages of introducing technologies in the manufacturing and distribution levels using RFID. This is even more important as drugs that are highly recognized and are in high demand in the marketplace receive less scrutiny. Technologies in large and small scale logistics could aid in faster and autonomous verification. “Good counterfeiters” invest a lot of time and effort into packaging in order to go undetected or arouse less suspicion. As labelling is the first area approached by counterfeiters it is even more important that small scale technologies are innovated and implemented into packaging.

These are some examples of the types of drugs that are being falsely labelled, Avastin (for cancer treatment) in the United States in 2012, made its way onto the market and affected 19 medical practices. Investigation confirmed that the drugs contained no active ingredient. Truvada and Viread (for HIV/AIDS) was seized in the United Kingdom in 2011, again false labelling was used and in Kenya in 2011, just under 3,000 patients were affected by counterfeit Zidolam-N (for HIV/AIDS). This again highlights the even more importance of implementing technologies that can reduce fake labelling and increase the potential to verify drugs more easily.

Further steps and checks should be made to ensure that packaging that is being disposed of, is destroyed in a manner, which will make it difficult to reuse. Another aspect of the challenge in the first stage is making the entry for counterfeiters even more difficult. As some manufacturing plants close, the equipment can be resold at a much lower cost, thus providing an opportunity for a much lower capital investment to start a counterfeit operation. One method to tackle this could be to require a form of registration for all manufacturing equipment that can be used for pharmaceutical manufacturing, similar to car registration. We can agree that even in the auto industry it has its vulnerabilities but a record keeping system in itself could be a deterrent or aid in future investigations.

As discussed in this thesis, the Internet has become a big opportunity for fake pharmacies to sell counterfeit and substandard drugs. Consumers do not know how to verify if the vendors are authentic or if they are reputable. The drugs being delivered could be shipped from countries that have a high probability of counterfeit or substandard drug track record and low regulation from authorities. Consumers are looking for alternatives to their current suppliers, as cost reduction is one of the biggest incentives. They do not care who supplies them the drugs as long as they believe them to be authentic and at a cheaper price. Criminals are always looking for the most profitable opportunities. Counterfeiters focused more on supplying individuals with lifestyle drugs but some are now shifting their focus to supplying pharmaceutical wholesalers, prioritizing those that supply aid organizations and hospitals. These are the ones that focus heavily on cost reduction. For counterfeiters to exploit this is very easy. The lack or no regulation in fake online pharmacies makes it extremely difficult for authorities to crack down on these global operations. With the growing popularity of unregulated virtual currencies such as Bitcoin and consumers accepting the use of virtual currencies by using intermediary merchants to convert and pay for items online, it is becoming more difficult for authorities to track monetary transactions online.

Free trade has opened up international markets for developing countries to export key pharmaceuticals. Unfortunately as discussed, due to low regulations and standards, this has opened up the risk of counterfeit and substandard production. Local authorities in developing countries could choose local economic developments over a global initiative to fight drug counterfeiting. Another challenge to tackle is on the border entry points, if customs cannot authenticate in the field they must allow drugs to continue on their destined route. Improvement in detection and testing at key import zones could increase the quantity of counterfeit and substandard drugs being seized. The next stage of detection needs to be held at the distribution level, where those who wish to trade pharmaceuticals to smaller regional distributors need to accept some form of responsibility and accountability for drugs that are being handled. The last phase of verification and detection must be at the dispensing stage. We can agree that system updates across many levels will come at a cost, especially in a time where healthcare systems are under a lot of financial pressure, but the future savings and risk reduction is worth the investment and future savings.

Additional methods for detecting counterfeit and substandard medicines could be by using Physical Chemical Identifiers (PCiDs). According to the FDA, PCiD is a substance or combination of substances possessing a unique physical or chemical property that unequivocally identifies and authenticates a drug product or dosage form (Food and Drug Administration, 2011a). As machinery is more expensive and there are difficulties in certain techniques, such as colour matching when layering, this could deter counterfeiters to even attempt. As new methods and techniques in the manufacturing process are implemented, a new level of intricacy such as compression techniques for embossing and layering, to printing logos and bar codes, make it more difficult for counterfeiters. This in itself adds a critical element for the end consumer, increasing the chances of detection before consumption. Another identifiable PCiD technique is the use of pearlescent colours in the film coating process that make it very hard to replicate the colours. These predominantly rely on the consumer being aware of their drug/s, especially with taste as some drugs are coated with flavours to mask the taste of the core tablet. However adding an element of taste and appearance does have its drawbacks such as higher costs. With the added machinery and manufacturing processes, these costs will undoubtedly be passed onto the consumer, but these extra costs could be subsidised by regulators or governments.

There are the additional factors in film coating techniques, such as Micro Taggants, that are microscopic particles, etched onto the drug, but this form factor in my opinion is not as effective as labelling technology. Because verification requires inspection of an actual tablet using a field reader, this would mean that only drugs that have raised suspicion would be tested (Zadbuke et al., 2013).

As mentioned in the thesis here is another example on the slow to react and implement of important changes to regulation and anti-counterfeiting measures. The falsified medicines directive (FMD) was published back in 2011 with a goal to successfully implement it by 2018 (European Commission, 2011). Seven years to implement such measures is far too long. Especially with the rate of speed that counterfeiters operate and adapt, this is unacceptable. Laws need to be tightened to put consumer safety as the first priority and if pharmaceutical companies do not comply, heavy fines should be imposed.

One of the solutions to assist in detecting counterfeit and substandard medications could be to install testing facilities within hospitals staffed with trained personnel. Another solution could be independent analytical units within a region or a city as is the case in the U.K. with food safety testing. These units could random test those medications that routinely raise suspicion about their quality or effectiveness. This could provide an additional layer of safety before reaching patients. Additionally, it can indicate when counterfeits have entered the supply chain. The costs to implement such analytical units could be high but at least it would protect patients and reduce further complications. These units would be independent from regulators and drug companies removing any conflict of interest.

With the evidence from the USA of former government employees taking senior positions in large pharmaceutical companies, it is clear that a conflict of interest is present. This could be a reason why regulations have never been put in place or are too relaxed, and are not challenged. This is despite the benefits of regulation outweighing the risks. One example is the link between the former U.S. Secretary of Defence and the approval of the sugar substitute “Aspartame”. When a suspicion of a conflict of interest is raised, this needs to be investigated and addressed. You can always follow the money and there is no smoke without fire. With big pharmaceutical companies donating millions of dollars to political campaigns, these serve as an IOU for later years and consumers may no longer be a priority (Gennet, 2011).

7.6 Possible further studies

7.6.1 Ciclosporin

1. Analyse more capsules from a wider range of countries so adding to the existing list of generics.

2. Improve the method to investigate more impurities in both the generic and brand capsules.

3. Investigation of possible toxic effect from impurities in all capsules.

7.6.2 Other drugs

1. Set up a new study to measure the active ingredients of lisinopril in brand versus generic tablets obtained from different countries.

2. This will involve development of a nonspecific capillary electrophoresis method and a HPLC method for determination of active ingredients of lisinopril in tablets.

3. Development of a methodology that will enable us to accurately identify the impurities in both ciclosporin and lisinopril. This will involve development of either LC-MS or NMR methodology for identification of impurities

4. Set up a pharmacokinetic study in patients receiving brand versus generic ciclosporin and lisinopril formulations in order to accurately measure the efficacy/toxicity profile and the bioequivalence of the generic formulations

Alfazema & Perrett (1997) developed, using chemometrics, a generic MECC method to analyse complex urines and other unknowns (Alfazema et al., 1997). Cyclodextrins (CDs) are powerful modifiers of CE separations. With low-wavelength UV, sulphated-ß-CD-MECC can separate and detect both charged and uncharged species simultaneously

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[1] Due to the nature of the counterfeit challenges, many of the references were only obtainable from regulatory health organisation websites such as the FDA, EMA, MHRA and WHO.

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Appendices

Appendix 1: Raw Data for ciclosporin HPLC test A and B

Table A1.1: Peak areas for ciclosporin brand S

Sample	Peak Area	Ciclosporin mg/mL
S1 5 min	0	0.00
S1 10 min	68.3	0.15
S1 15 min	85.1	0.19
S1 30 min	87.2	0.19
S1 60 min	83.9	0.18
S1 90 min	82.2	0.18
S2 5 min	0	0.00
S2 10 min	36.1	0.08
S2 15 min	72.5	0.16
S2 30 min	85.7	0.19
S2 60 min	88.1	0.19
S2 90 min	85	0.19
S3 5 min	0	0.00
S3 10 min	46.8	0.10
S3 15 min	73.5	0.16
S3 30 min	84.8	0.19
S3 60 min	80.5	0.18
S3 90 min	86.2	0.19
S4 5 min	1.4	0.00
S4 10 min	63.8	0.14
S4 15 min	88.4	0.19
S4 30 min	91.4	0.20
S4 60 min	87	0.19
S4 90 min	97.4	0.21

Table A1.2: Peak areas for ciclosporin brand T

Sample	Peak Area	Ciclosporin mg/mL
T1 5 min	17.3	0.04
T1 10 min	54.6	0.13
T1 15 min	73.8	0.18
T1 30 min	85.8	0.21
T1 60 min	86	0.21
T1 90 min	91.5	0.22
T2 5 min	0	0.00
T2 10 min	62.1	0.15
T2 15 min	95.3	0.23
T2 30 min	86.6	0.21
T2 60 min	88.5	0.22
T2 90 min	87.6	0.21
T3 5 min	4.8	0.01
T3 10min	70.4	0.17
T3 15 min	77.2	0.19
T3 30 min	81.9	0.20
T3 60 min	80	0.20
T3 90 min	88.4	0.22
T4 5 min	0	0.00
T4 10 min	0	0.00
T4 15 min	86.5	0.21
T4 30 min	83.2	0.20
T4 60 min	87.1	0.21
T4 90 min	81.9	0.20

Table A1.3: Peak areas for ciclosporin brand P

Sample	Peak Area	Ciclosporin mg/mL
P1 5 min	0	0.00
P1 10 min	73.5	0.16
P1 15 min	80.8	0.18
P1 30 min	84	0.19
P1 60 min	87	0.19
P1 90 min	82.5	0.18
P2 5 min	0	0.00
P2 10 min	67.5	0.15
P2 15 min	96.2	0.21
P2 30 min	72.8	0.16
P2 60 min	90.2	0.20
P2 90 min	88.3	0.20
P3 5 min	0	0.00
P3 10 min	71.6	0.16
P3 15 min	85.3	0.19
P3 30 min	82.2	0.18
P3 60 min	82.9	0.18
P3 90 min	89.6	0.20
P4 5 min	25.2	0.06
P4 10 min	80.4	0.18
P4 15 min	86.4	0.19
P4 30 min	98.2	0.22
P4 60 min	88.3	0.20
P4 90 min	88.1	0.19

Table A1.4: Peak areas for ciclosporin brand J

Sample	Peak Area	Ciclosporin mg/mL
J1 5 min	2.3	0.00
J1 10 min	28.4	0.06
J1 15 min	32.6	0.07
J1 30 min	39.9	0.09
J1 60 min	42.3	0.09
J1 90 min	41.7	0.09
J2 5 min	0	0.00
J2 10 min	30.8	0.07
J2 15 min	38.5	0.08
J2 30 min	38.7	0.08
J2 60 min	39.6	0.09
J2 90 min	37.3	0.08
J3 5 min	1.8	0.00
J3 10 min	34.2	0.07
J3 15 min	37.6	0.08
J3 30 min	38.9	0.08
J3 60 min	40.4	0.09
J3 90 min	41	0.09
J4 5 min	1.8	0.00
J4 10 min	29.6	0.06
J4 15 min	39.1	0.08
J4 30 min	37.4	0.08
J4 60 min	41.3	0.09
J4 90 min	42.5	0.09

Table A1.5: Peak areas for ciclosporin brand E

Sample	Peak Area	Ciclosporin mg/mL
E1 5 min	0	0.00
E1 10 min	3.3	0.01
E1 15 min	18.9	0.04
E1 30 min	32	0.07
E1 60 min	35.7	0.08
E1 90 min	40.5	0.09
E2 5 min	1.9	0.00
E2 10 min	0	0.00
E2 15 min	18.2	0.04
E2 30 min	34.3	0.07
E2 60 min	35.2	0.08
E2 90 min	37.5	0.08
E3 5 min	1.9	0.00
E3 10 min	8.8	0.02
E3 15 min	35.5	0.08
E3 30 min	37.6	0.08
E3 60 min	37.1	0.08
E3 90 min	40.2	0.09
E4 5 min	1.9	0.00
E4 10 min	13.3	0.03
E4 15 min	33.5	0.07
E4 30 min	40	0.09
E4 60 min	40.1	0.09
E4 90 min	41.7	0.09

Table A1.6: Peak areas for ciclosporin generic C

Sample	Peak Area	Ciclosporin mg/mL
C1 5 min	68.8	0.15
C1 10 min	82.5	0.18
C1 15 min	80.8	0.18
C1 30 min	85	0.19
C1 60 min	86.1	0.19
C1 90 min	88.5	0.19
C2 5 min	79.7	0.18
C2 10 min	82.6	0.18
C2 15 min	84.6	0.19
C2 30 min	83.8	0.18
C2 60 min	76.2	0.17
C2 90 min	86.1	0.19
C3 5 min	75.6	0.17
C3 10 min	96.7	0.21
C3 15 min	81.9	0.18
C3 30 min	87.6	0.19
C3 60 min	86.8	0.19
C3 90 min	87.7	0.19
C4 5 min	89.2	0.20
C4 10 min	90.8	0.20
C4 15 min	83.5	0.18
C4 30 min	83.8	0.18
C4 60 min	81.5	0.18
C4 90 min	88.8	0.20

Table A1.7: Peak areas for ciclosporin generic I

Sample	Peak Area	Ciclosporin mg/mL
I1 5 min	16.9	0.04
I1 10 min	60	0.13
I1 15 min	68	0.15
I1 30 min	69.7	0.15
I1 60 min	71.1	0.16
I1 90 min	61.8	0.14
I2 5 min	0	0.00
I2 10 min	46.9	0.10
I2 15 min	67.4	0.15
I2 30 min	60.2	0.13
I2 60 min	66.1	0.15
I2 90 min	66.9	0.15
I3 5 min	0	0.00
I3 10 min	54.1	0.12
I3 15 min	65.2	0.14
I3 30 min	59.7	0.13
I3 60 min	63.4	0.14
I3 90 min	61.4	0.14
I4 5 min	0.1	0.00
I4 10 min	15.9	0.04
I4 15 min	68.6	0.15
I4 30 min	58.4	0.13
I4 60 min	62.2	0.14
I4 90 min	77.2	0.17

Table A1.8: Ciclosporin capsules rupture times test A

Ciclosporin	Rupture time (min)				Average (min)
	Run 1	Run 2	Run 3	Run 4	
Brand S	05:30	05:49	06:20	05:13	05:43:00
Brand T	05:00	05:16	04:30	05:42	05:07:00
Brand P	06:00	06:00	05:30	04:59	05:37:15
Brand J	05:10	04:54	05:23	05:19	05:11:30
Brand E	06:00	05:27	05:31	05:01	05:29:45
Generic C	02:20	01:10	02:15	02:00	01:56:15
Generic I	04:40	05:50	06:22	05:27	05:34:45

Table A1.9: Peak areas for ciclosporin brand Sa

Sample	Peak Area	Ciclosporin mg/L
Sa1 5 min	217.6	4
Sa1 10 min	5557.5	100
Sa1 15 min	8385.6	151
Sa1 20 min	9144.8	164
Sa1 30 min	9187.7	165
Sa1 45 min	9739.5	175
Sa1 60 min	9595.2	172
Sa1 90 min	9756.6	175
Sa1 120 min	10061.6	181
Sa2 5 min	252.1	5
Sa2 10 min	4906	88
Sa2 15 min	6541.1	118
Sa2 20 min	7204.2	129
Sa2 30 min	8350.5	150
Sa2 45 min	9647.7	173
Sa2 60 min	9576.8	172
Sa2 90 min	10217.7	184
Sa2 120 min	10228.7	184
Sa3 5 min	226	4
Sa3 10 min	7248.4	130
Sa3 15 min	8442.8	152
Sa3 20 min	8625.6	155
Sa3 30 min	9047.7	163
Sa3 45 min	8785.9	158
Sa3 60 min	9534.1	171
Sa3 90 min	9932	179
Sa3 120 min	9875.3	177
Sa4 5 min	251.7	5
Sa4 10 min	5574.8	100
Sa4 15 min	7615.3	137
Sa4 20 min	9181	165
Sa4 30 min	9368.6	168
Sa4 45 min	9849	177
Sa4 60 min	10076.4	181
Sa4 90 min	9840.4	177
Sa4 120 min	10043	181
Sa5 5 min	0	0
Sa5 10 min	1723.3	31
Sa5 15 min	2692.6	48
Sa5 20 min	7984.8	144
Sa5 30 min	8507.3	153
Sa5 45 min	8917	160
Sa5 60 min	9483.9	170
Sa5 90 min	10119.6	182
Sa5 120 min	10235.8	184

Table A1.10: Peak areas for ciclosporin brand TK

Sample	Peak Area	Ciclosporin mg/L
TK1 5 min	0.6	0
TK1 10 min	1969.1	36
TK1 15 min	4396.6	80
TK1 20 min	7627.4	138
TK1 30 min	8528	154
TK1 45 min	9132.4	165
TK1 60 min	9711.4	176
TK1 90 min	9919.2	180
TK1 120 min	10105.5	183
TK2 5 min	1503.4	27
TK2 10 min	6155.8	111
TK2 15 min	7105.1	129
TK2 20 min	7966.5	144
TK2 30 min	8651.7	157
TK2 45 min	8866.2	161
TK2 60 min	8675.2	157
TK2 90 min	9448.3	171
TK2 120 min	10041.6	182
TK3 5 min	7.4	0
TK3 10 min	7552.9	137
TK3 15 min	8819.3	160
TK3 20 min	9494.5	172
TK3 30 min	9709.2	176
TK3 45 min	9910.6	179
TK3 60 min	10097	183
TK3 90 min	10144.5	184
TK3 120 min	10204.6	185
TK4 5 min	174.1	3
TK4 10 min	7876.7	143
TK4 15 min	9586.8	174
TK4 20 min	9628.5	174
TK4 30 min	9801	178
TK4 45 min	9860.8	179
TK4 60 min	9960	180
TK4 90 min	10069.5	182
TK4 120 min	9955	180
TK5 5 min	217.3	4
TK5 10 min	5395.6	98
TK5 15 min	7129.8	129
TK5 20 min	8662.3	157
TK5 30 min	9498.5	172
TK5 45 min	9680.1	175
TK5 60 min	9885.6	179
TK5 90 min	9954.2	180
TK5 120 min	9969.9	181

Table A1.11: Peak areas for ciclosporin brand Pak

Sample	Peak Area	Ciclosporin mg/L
Pak1 5 min	0	0
Pak1 10 min	6561.1	117
Pak1 15 min	8749.6	156
Pak1 20 min	9065.9	161
Pak1 30 min	9318.2	166
Pak1 45 min	9707.6	173
Pak1 60 min	9896.9	176
Pak1 90 min	10044.5	179
Pak1 120 min	10122.4	180
Pak2 5 min	164.5	3
Pak2 10 min	7312.5	130
Pak2 15 min	8994	160
Pak2 20 min	9208.7	164
Pak2 30 min	9536.7	170
Pak2 45 min	9861	176
Pak2 60 min	9910.1	176
Pak2 90 min	10106.8	180
Pak2 120 min	9997.7	178
Pak3 5 min	180.6	3
Pak3 10 min	7092.6	126
Pak3 15 min	7830.3	139
Pak3 20 min	8264.7	147
Pak3 30 min	8843.8	157
Pak3 45 min	9107.6	162
Pak3 60 min	9264.1	165
Pak3 90 min	9722.9	173
Pak3 120 min	9731.5	173
Pak4 5 min	208.4	4
Pak4 10 min	6731.3	120
Pak4 15 min	8319.9	148
Pak4 20 min	8704.6	155
Pak4 30 min	9226.3	164
Pak4 45 min	9594.9	171
Pak4 60 min	9666.3	172
Pak4 90 min	9536.8	170
Pak4 120 min	9728.6	173
Pak5 5 min	338.3	6
Pak5 10 min	6420	114
Pak5 15 min	7553.7	134
Pak5 20 min	8110	144
Pak5 30 min	8554.1	152
Pak5 45 min	9007.2	160
Pak5 60 min	9078	162
Pak5 90 min	9350.4	166
Pak5 120 min	8782.3	156

Table A1.12: Peak areas for ciclosporin brand Jor

Sample	Peak Area	Ciclosporin mg/L
Jor1 5 min	289.1	5
Jor1 10 min	3550.2	64
Jor1 15 min	4312.4	78
Jor1 20 min	3976.1	72
Jor1 30 min	3902.6	70
Jor1 45 min	3512.4	63
Jor1 60 min	3431.5	62
Jor1 90 min	3448.9	62
Jor1 120 min	3372.5	61
Jor2 5 min	283.5	5
Jor2 10 min	4128.2	74
Jor2 15 min	3604.8	65
Jor2 20 min	3603.3	65
Jor2 30 min	3455.9	62
Jor2 45 min	3483.5	63
Jor2 60 min	4437	80
Jor2 90 min	4516.1	81
Jor2 120 min	4592.7	83
Jor3 5 min	289.3	5
Jor3 10 min	3241.7	58
Jor3 15 min	3867.6	70
Jor3 20 min	4238.5	76
Jor3 30 min	4461.4	80
Jor3 45 min	4508.9	81
Jor3 60 min	4438.3	80
Jor3 90 min	4560.5	82
Jor3 120 min	4600.5	83
Jor4 5 min	303.6	5
Jor4 10 min	3286.7	59
Jor4 15 min	3783.9	68
Jor4 20 min	3971.9	72
Jor4 30 min	3966.2	71
Jor4 45 min	3814	69
Jor4 60 min	3879.5	70
Jor4 90 min	4054.3	73
Jor4 120 min	4353.7	78
Jor5 5 min	292.2	5
Jor5 10 min	1685.5	30
Jor5 15 min	3125.2	56
Jor5 20 min	3436.1	62
Jor5 30 min	3605.9	65
Jor5 45 min	3920.4	71
Jor5 60 min	4316.8	78
Jor5 90 min	4324.6	78
Jor5 120 min	4457.1	80

Table A1.13: Peak areas for ciclosporin brand Egy

Sample	Peak Area	Ciclosporin mg/L
Egy1 5 min	258.9	5
Egy1 10 min	582.1	11
Egy1 15 min	3431.5	62
Egy1 20 min	4112.8	75
Egy1 30 min	4234.9	77
Egy1 45 min	4347.8	79
Egy1 60 min	4477.4	81
Egy1 90 min	4602.6	84
Egy1 120 min	4577	83
Egy2 5 min	254.1	5
Egy2 10 min	326.6	6
Egy2 15 min	3195.1	58
Egy2 20 min	3512.8	64
Egy2 30 min	3922.8	71
Egy2 45 min	4189.7	76
Egy2 60 min	4205.9	76
Egy2 90 min	4541.2	82
Egy2 120 min	4593.2	83
Egy3 5 min	258.2	5
Egy3 10 min	1539.8	28
Egy3 15 min	3770.2	68
Egy3 20 min	4177.4	76
Egy3 30 min	4196.3	76
Egy3 45 min	4459.9	81
Egy3 60 min	4398.4	80
Egy3 90 min	4526.8	82
Egy3 120 min	4628.4	84
Egy4 5 min	265	5
Egy4 10 min	329.2	6
Egy4 15 min	2349.5	43
Egy4 20 min	3579.9	65
Egy4 30 min	4000.1	73
Egy4 45 min	4233.7	77
Egy4 60 min	4454.7	81
Egy4 90 min	4633.7	84
Egy4 120 min	4569.9	83
Egy5 5 min	266.8	5
Egy5 10 min	569.5	10
Egy5 15 min	3135.3	57
Egy5 20 min	3674.9	67
Egy5 30 min	3921.4	71
Egy5 45 min	4256.9	77
Egy5 60 min	4304.2	78
Egy5 90 min	4465.7	81
Egy5 120 min	4435.4	81

Table A1.14: Peak areas for ciclosporin generic Col

Sample	Peak Area	Ciclosporin mg/L
Col1 5 min	7958.6	143
Col1 10 min	8410.6	151
Col1 15 min	8333.4	150
Col1 20 min	8283.9	149
Col1 30 min	8635.8	155
Col1 45 min	8642.3	155
Col1 60 min	8660.9	156
Col1 90 min	8741.8	157
Col1 120 min	8456.9	152
Col2 5 min	6094.7	109
Col2 10 min	8103	145
Col2 15 min	8429	151
Col2 20 min	8669.9	156
Col2 30 min	8668.8	156
Col2 45 min	8603.8	154
Col2 60 min	8799	158
Col2 90 min	8616.9	155
Col2 120 min	8513.7	153
Col3 5 min	7872.5	141
Col3 10 min	8245.2	148
Col3 15 min	8450.5	152
Col3 20 min	8195.4	147
Col3 30 min	8460.3	152
Col3 45 min	8570	154
Col3 60 min	8545.8	153
Col3 90 min	8453.9	152
Col3 120 min	8332.2	150
Col4 5 min	8056.1	145
Col4 10 min	8208.9	147
Col4 15 min	8353.5	150
Col4 20 min	8330.8	150
Col4 30 min	8409	151
Col4 45 min	8275.1	149
Col4 60 min	8304.9	149
Col4 90 min	8277.2	149
Col4 120 min	8224	148
Col5 5 min	7674.8	138
Col5 10 min	8218	148
Col5 15 min	8219.8	148
Col5 20 min	8298.6	149
Col5 30 min	8413.4	151
Col5 45 min	8434.5	151
Col5 60 min	8755.1	157
Col5 90 min	8667.1	156
Col5 120 min	8534.7	153

Table A1.15: Peak areas for ciclosporin generic Ir

Sample	Peak Area	Ciclosporin mg/L
Ir1 5 min	366.8	7
Ir1 10 min	3152.5	56
Ir115 min	6124.8	109
Ir1 20 min	7878.4	141
Ir1 30 min	9249.7	165
Ir1 45 min	9853.5	176
Ir1 60 min	10151.9	181
Ir1 90 min	9747.7	174
Ir1 120 min	9751.2	174
Ir2 5 min	10.3	0
Ir2 10 min	3313.6	59
Ir2 15 min	6149.1	110
Ir2 20 min	7818.6	140
Ir2 30 min	8939.5	160
Ir2 45 min	9807.8	175
Ir2 60 min	10078.5	180
Ir2 90 min	9943.3	178
Ir2 120 min	9973.5	178
Ir3 5 min	390.1	7
Ir3 10 min	4886.9	87
Ir3 15 min	7500	134
Ir3 20 min	8265.8	148
Ir3 30 min	8755.5	156
Ir3 45 min	9388.4	168
Ir3 60 min	9828	176
Ir3 90 min	9991.8	179
Ir3 120 min	9750.5	174
Ir4 5 min	340.1	6
Ir4 10 min	3608.5	64
Ir4 15 min	6027.6	108
Ir4 20 min	7239.7	129
Ir4 30 min	9026	161
Ir4 45 min	9723.4	174
Ir4 60 min	9665	173
Ir4 90 min	9557.8	171
Ir4 120 min	9597.9	172
Ir5 5 min	277.3	5
Ir5 10 min	5983.9	107
Ir5 15 min	8403.2	150
Ir5 20 min	9115.3	163
Ir5 30 min	9429.6	169
Ir5 45 min	9529.1	170
Ir5 60 min	9652.4	173
Ir5 90 min	9521.7	170
Ir5 120 min	9601.7	172

Table A1.16: Peak areas for ciclosporin generic M

Sample	Peak Area	Ciclosporin mg/L
M1 5 min	5.4	0
M1 10 min	5687.5	103
M1 15 min	5660.8	103
M1 20 min	6160.2	112
M1 30 min	5998.1	109
M1 45 min	7397.4	135
M1 60 min	7680.4	140
M1 90 min	6545.9	119
M1 120 min	6745.4	123
M2 5 min	4	0
M2 10 min	3015.6	55
M2 15 min	5786.5	105
M2 20 min	7081.1	129
M2 30 min	6574.3	120
M2 45 min	6780.6	123
M2 60 min	6411.5	117
M2 90 min	5214.6	95
M2 120 min	5783.2	105
M3 5 min	0.3	0
M3 10 min	4289.5	78
M3 15 min	5204.2	95
M3 20 min	4915.1	89
M3 30 min	5579	102
M3 45 min	4679.4	85
M3 60 min	5302	96
M3 90 min	4831.1	88
M3 120 min	4972.6	90
M4 5 min	2.8	0
M4 10 min	2095	38
M4 15 min	3592.7	65
M4 20 min	3640.4	66
M4 30 min	3306.8	60
M4 45 min	3387.4	62
M4 60 min	3862.3	70
M4 90 min	3890.3	71
M4 120 min	4652	85
M5 5 min	3.5	0
M5 10 min	382.3	7
M5 15 min	3658.6	67
M5 20 min	4561.8	83
M5 30 min	4992.9	91
M5 45 min	5239	95
M5 60 min	5150.5	94
M5 90 min	6040.4	110
M5 120 min	5699.7	104

Table A1.17: Ciclosporin capsules rupture times test B

Ciclosporin	Rupture time (min)				
	Run 1	Run 2	Run 3	Run 4	Run 5
Brand Sa	All capsules ruptured within 15 min				
Brand TK	All capsules ruptured within 15 min				
Brand Pak	All capsules ruptured within 15 min				
Brand Jor	All capsules ruptured within 15 min				
Brand Egy	All capsules ruptured within 15 min				
Generic Col	All capsules ruptured within 15 min				
Generic Ir	All capsules ruptured within 15 min				
Generic M	All capsules ruptured within 15 min				

Appendix 2: UHPLC-MS results of the impurities detection in ciclosporin capsules

Table A2.18: Impurities in ciclosporin capsules from Colombia (Col), in negative and positive modes

Col Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C6H14O6	Sorbitol	261.17481	8.9E-07
C18H34O3	2-Oxooctadecanoic acid	26.9869983	0.00135
C7H13O6P	Mevinphos	2.08953018	0.46759
C10H6N2O2S2	Oxidized Photinus luciferin	1.97221459	0.00238
C5H10O6	D-Xylonate	1.57202829	0.40148
Col Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C33H49N5O6	Zizyphine A	3409.64127	6.95E-10
C26H41NO7	Delcorine	1077.2487	3.5E-09
Br3GaH3N	gallium;azane;tribromide	952.8728	3.4E-09
C24H51NO7P	1-Palmitoylglycerophosphocholine	841.99466	1E-09
C26H45N7O7	seryl-HYPE(transpropyl)-octahydroindole-2-carbonyl-arginine	618.967749	1.3E-06

Table A2.19: Impurities in ciclosporin capsules from Egypt (Egy), in negative and positive modes

Eg Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
I4In-	tetraiodoindiganuide	1688.45986	0.34626
C17H35NO3	AC1L4KW4	54.0655596	0.33662
C17H35NO3	AC1L4KW4	52.4486712	0.33574
C20H40O3	L-2-Hydroxyphytanate	29.7710108	0.3363
C18H32O3	(9Z,12Z)-(8R)-Hydroxyoctadeca-9,12-dienoic acid	17.6520245	0.32446
Eg Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C20H34O3	2alpha-(Hydroxymethyl)-5alpha-androstane-3beta,17beta-diol	10.0637702	0.34073
C36H74Hg	Dioctadecylmercury	8.69145162	0.39697
C7H10O	3-CYCLOHEXENE-1-CARBOXALDEHYDE	8.47751377	0.0001
C20H32O3	(15S)-15-Hydroxy-5,8,11-cis-13-trans-eicosatetraenoate	7.89162577	0.27577
C25H32N2O2	Dextromoramide	5.26814004	0.29427

Table A2.20: Impurities in ciclosporin capsules from India (I), in negative and positive modes

India Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C29H30O8	Piperaduncin B	3506.41425	8.5E-08
C12H24O11	Melibiitol	1341.30713	1.8E-10
C22H21N2S+	AC1L2KWH	1224.92981	7.1E-10
C21H21ClN4OS	Ziprasidone	606.023622	1.6E-08
C6H14O6	Sorbitol	90.784177	3.3E-09
India Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
Cl6H2Pt	chloroplatinic acid	2593.85547	2.6E-10
C12H24O2	lauric acid	2069.97359	1.7E-08
C15H25Cl3N4	N-ethyl-4-nonyl-6-(trichloromethyl)-1,3,5-triazin-2-amine	1436.77562	1.7E-10
C10H23N3O	N,N-Dibutylglycine hydrazide	1418.85452	1E-06

Table A2.21: Impurities in ciclosporin capsules from Iran (Ir), in negative and positive modes

Ir Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C6H14O6	Sorbitol	497.818238	1.1E-11
C12H24O11	Melibiitol	262.2803	2E-08
C9H12N2O5	Deoxyuridine	100.136193	2.5E-14
C7H13O6P	Mevinphos	3.41571746	0.21911
C10H6N2O2S2	Oxidized Photinus luciferin	1.73012836	0.06219
Ir Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C38H63NO10	alpha-(3-hydroxysialyl)cholesterol	9887.12843	3.1E-07
C37H63NO6S	AC1L5827	5180.27028	1.2E-06
C31H62NO8P	PC(10:0/13:0)[U]	5179.92812	0.00051
C35H60O7P2	all-trans-Heptaprenyl diphosphate	4168.99115	4E-07
C26H41NO7	Delcorine	3007.32626	2.3E-05
C40H63NO6	NSC679741	2297.89493	0.00387

Table A2.22: Impurities in ciclosporin capsules from Jordan (Jor), in negative and positive modes

Jor Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C18H36O3	**(R)-2-Hydroxystearate**	**3.06488194**	**0.04801**
C18H36O3	**(R)-2-Hydroxystearate**	**2.00790836**	**0.17774**
C10H6N2O2S2	**Oxidized Photinus luciferin**	**1.82755613**	**0.05196**
C10H6N2O2S2	**Oxidized Photinus luciferin**	**1.42473336**	**1E-05**
C19H24N2O2	**Praziquantel**	**1.27067976**	**0.55117**
Jor Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C6H9N3	**3,3'-iminodipropionitrile**	**3.3215266**	**0.01494**
C4H5NO	**allyl isocyanate**	**2.65591664**	**0.00167**
C20H36O3	**15-hydroxyicosa-11,13-dienoic acid**	**2.63146847**	**3.9E-05**
C20H36O3	**15-hydroxyicosa-11,13-dienoic acid**	**2.37246828**	**0.00049**
FO3P-2	**fluorophosphate**	**1.70247524**	**0.00037**

Table A2.23: Impurities in ciclosporin capsules from Morocco (M), in negative and positive modes

M Neg			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C41H54O2	**Okenone**	**11839.3048**	**5.5E-06**
C42H56O4	**2,2'-Diketospirilloxanthin**	**5986.36865**	**3.1E-05**
C11H20O10	**Vicianose**	**5552.06902**	**0.34668**
C34H50N2O4+2	**AC1L1RH6**	**4665.35719**	**7.3E-05**
H2O7P2-2	**pyrophosphate 2-**	**2250.24899**	**0.3468**
C26H52N2O5S	**61741-09-1**	**1515.07028**	**7.8E-07**
Tc	**Technetium-101**	**840.783931**	**0.34646**
M Pos			
Molecular formula	**Name**	**Fold Change**	**Ttest**
C30H46N4O4	**AC1L1G95**	**1522.08278**	**0.00032**
C38H52N2O4+2	**AC1L46ED**	**1303.11028**	**0.00018**
C44H87NO8P+	**AC1MHWLG**	**1256.02653**	**0.10026**
C29H48O7	**Contignasterol**	**793.070597**	**0.00054**
C40H46N4	**BRN 0466832**	**743.569797**	**0.00027**
C42H83NO7P	**1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphonocholine**	**638.292384**	**0.11432**
C41H54O2	**Okenone**	**630.807688**	**0.00031**
C27H25Br5N2O9	**CHEMBL454956**	**570.038657**	**0.23225**
C31H70O18	**AC1L1WNS**	**449.927854**	**0.00025**

Table A2.24: Impurities in ciclosporin capsules from Pakistan (Pak), in negative and positive modes

Pak Neg			
Molecular formula	Name	Fold Change	Ttest
C10H6N2O2S2	Oxidized Photinus luciferin	1.07840377	0.22804
C18H36N6O4	Ile Arg Ile	1.06317935	0.47937
C10H6N2O2S2	Oxidized Photinus luciferin	1.02430833	0.86963
C20H24O3	Acitretin Ro 12-7310	1.01927338	0.87279
C21H26O3	Acitretin	0.99859995	0.98356
C18H38N6O4	Lys Lys Lys	0.99355099	0.93072
C14H22N2O3	Atenolol	0.98993979	0.91512
C18H22N2S	Trimeprazine	0.97062822	0.44611
C4H2O4	Acetylenedicarboxylate	0.96777327	0.55454
Pak			
Molecular formula	Name	Fold Change	Ttest
C22H43NO	erucyl amide	320.773752	0.13787
C20H40N2. C2H4O2	Glyodin	110.947191	0.06791
C32H64N4O2	AC1NMO0A	97.9016981	0.09702
C19H36O3	methyl ricinoleate	30.2515164	0.03053
C20H36O3	15-hydroxyicosa-11,13-dienoic acid	4.60343945	1.9E-09
C20H36O3	15-hydroxyicosa-11,13-dienoic acid	4.01295774	1.7E-09
C27H22F4N4O3S	Losulazine	1.75328667	0.31099
C5H8O3S	4-Methylthio-2-oxobutanoic acid	1.72723948	0.08443

Table A2.25: Impurities in ciclosporin capsules from Saudi (Sa), in negative and positive modes

Sa Neg			
Molecular formula	Name	Fold Change	Ttest
C20H36O3	(±)11-HEDE	2.90547033	1.6E-05
C6H9N3	3,3'-iminodipropionitrile	2.64516157	0.08245
C18H28O	6-[5]-ladderane-1-hexanol	1.86989155	0.00018
C21H34O4	Tetrahydrocorticosterone	1.70920777	0.00086
Sa Pos		Fold Change	Ttest
C18H22O8P2	Diethylstilbestrol diphosphate	1.67917944	0.58649
C18H30O	4-Dodecylphenol	1.6715494	4.5E-07
C24H22O12	Malonyldaidzin	1.60361504	0.61524

Appendix 3: Metabolomic data analysis for ciclosporin capsules from Colombia (Col)

Metabolomic Data Analysis with MetaboAnalyst 2.0

User ID: guest121709985554323475

December 9, 2014

1 Data Processing and Normalization

1.1 Reading and Processing the Raw Data

MetaboAnalyst accepts a variety of data types generated in metabolomic studies, including compound concentration data, binned NMR/MS spectra data, NMR/MS peak list data, as well as MS spectra (NetCDF, mzXML, mzDATA). Users need to specify the data types when uploading their data in order for MetaboAnalyst to select the correct algorithm to process them. Table 1 summarizes the result of the data processing steps.

1.1.1 Reading MS Peak List and Intensities Data

MS peak list and intensities data should be uploaded as one zip file. It contains subfoulders with one for each group. Each folder contains peak list files, one per spectrum. The MS peak list format is either a two-column (mass and intensities) or three-column (mass, retention time, and intensities) comma separated values. The first line is assumed to be column labels. The files should be saved in .csv format. For paired analysis, users need to upload separately a text file specifying the paired information. Each pair is indicated by their sample names seperated by a colon ":" with one pair per line.

The uploaded files are peak lists and intensities data. A total of 10 samples were found. These samples contain a total of 3860 peaks. with an average of 386 peaks per sample

1.1.2 Peak Matching and Alignment

Peaks need to be matched across samples in order to be compared. For two-column data, the program matches peaks by their m/z values. For three-column data, the program will further group peaks based on their retention time. During the process, mz and rt of each peak will be changed to their group median values. If a sample has more than one peak in a group, they will be replaced by their sum. Some peaks are excluded if they appear in less than half of both classes. The aligned peaks are reorganized into a single data matrix for further analysis. The name of the parent folder is used as class label for each sample.

A total of 373 peak groups were formed. Peaks of the same group were summed if they are from one sample. Peaks appear in less than half of samples in each group were ignored.

1.1.3 Data Integrity Check

Before data analysis, a data integrity check is performed to make sure that all the necessary information has been collected. The class labels must be present and contain only two classes. If samples are paired, the class label must be from -n/2 to -1 for one group, and 1 to n/2 for the other group (n is the sample number and must be an even number). Class labels with same absolute value are assumed to be pairs.

Compound concentration or peak intensity values should all be non-negative numbers. By default, all missing values, zeros and negative values will be replaced by the half of the minimum positive value found within the data (see next section)

1.1.4 Missing value imputations

Too many zeroes or missing values will cause difficulties for downstream analysis. MetaboAnalyst offers several different methods for this purpose. The default method replaces all the missing and zero values with a small values (the half of the minimum positive values in the original data) assuming to be the detection limit. The assumption of this approach is that most missing values are caused by low abundance metabolites (i.e.below the detection limit). In addition, since zero values may cause problem for data normalization (i.e. log), they are also replaced with this small value. User can also specify other methods, such as replace by mean/median, or use K-Nearest Neighbours, Probabilistic PCA (PPCA), Bayesian PCA (BPCA) method, Singular Value Decomposition (SVD) method to impute the missing values [1]. Please choose the one that is the most appropriate for your data.

Zero or missing variables were replaced with a small value: 2.8153854225

1.1.5 Data Filtering

The purpose of the data filtering is to identify and remove variables that are unlikely to be of use when modeling the data. No phenotype information are used in the filtering process, so the result can be used with any downstream analysis. This step can usually improves the results. Data filter is strongly recommended for datasets with large number of variables (> 250) datasets contain much noise (i.e.chemometrics data). Filtering can usually improve your results[2].

For data with number of variables < 250, this step will reduce 5% of variables; For variable number between 250 and 500, 10% of variables will be removed; For variable number bwteen 500 and 1000, 25% of variables will be removed; And 40% of variabled will be removed for data with over 1000 varaibles.

No data filtering was performed.

Table 1: Summary of data processing results

	Peaks (raw)	Missing/Zero	Peaks (processed)
CoL01	386	0	373
CoL02	386	0	373
CoL03	386	0	373
CoL04	386	0	373
CoL05	386	0	373
TK_01	386	0	373
TK_02	386	0	373
TK_03	386	0	373
TK_04	386	0	373
TK_05	386	0	373

[1]Stacklies W, Redestig H, Scholz M, Walther D, Selbig J. *pcaMethods: a bioconductor package, providing PCA methods for incomplete data.*, Bioinformatics 2007 23(9):1164-1167

[2]Hackstadt AJ, Hess AM.*Filtering for increased power for microarray data analysis*, BMC Bioinformatics. 2009; 10: 11.

1.2 Data Normalization

The data is stored as a table with one sample per row and one variable (bin/peak/metabolite) per column. The normalization procedures implemented below are grouped into four categories. Sample specific normalization allows users to manually adjust concentrations based on biological inputs (i.e. volume, mass); row-wise normalization allows general-purpose adjustment for differences among samples; data transformation and scaling are two different approaches to make features more comparable. You can use one or combine both to achieve better results.

The normalization consists of the following options:

1. Sample specific normalization (i.e. normalize by dry weight, volume)
2. Row-wise procedures:
   - Normalization by the sum
   - Normalization by the sample median
   - Normalization by a reference sample (probabilistic quotient normalization)[3]
   - Normalization by a reference feature (i.e. creatinine, internal control)
3. Data transformation :
   - Generalized log transformation (glog 2)
   - Cube root transformation
4. Data scaling:
   - Unit scaling (mean-centered and divided by standard deviation of each variable)
   - Pareto scaling (mean-centered and divided by the square root of standard deviation of each variable)
   - Range scaling (mean-centered and divided by the value range of each variable)

Figure 1 shows the effects before and after normalization.

[3]Dieterle F, Ross A, Schlotterbeck G, Senn H. *Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics*, 2006, Anal Chem 78 (13);4281 - 4290

2 Statistical and Machine Learning Data Analysis

MetaboAnalyst offers a variety of methods commonly used in metabolomic data analyses. They include:

1. Univariate analysis methods:
   - Fold Change Analysis
   - T-tests
   - Volcano Plot
   - One-way ANOVA and post-hoc analysis
   - Correlation analysis
2. Multivariate analysis methods:
   - Principal Component Analysis (PCA)
   - Partial Least Squares - Discriminant Analysis (PLS-DA)
3. Robust Feature Selection Methods in microarray studies
   - Significance Analysis of Microarray (SAM)
   - Empirical Bayesian Analysis of Microarray (EBAM)
4. Clustering Analysis
   - Hierarchical Clustering
     - Dendrogram
     - Heatmap
   - Partitional Clustering
     - K-means Clustering
     - Self-Organizing Map (SOM)
5. Supervised Classification and Feature Selection methods
   - Random Forest
   - Support Vector Machine (SVM)

`Please note: some advanced methods are available only for two-group sample analyais.`

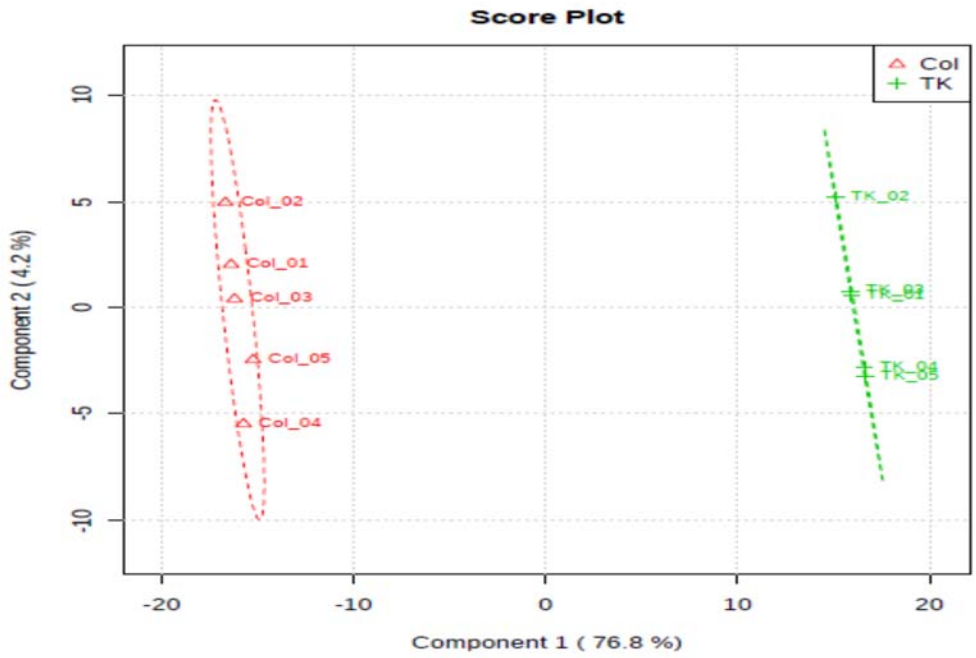

Figure A3.1: Score plot between the selected PCs

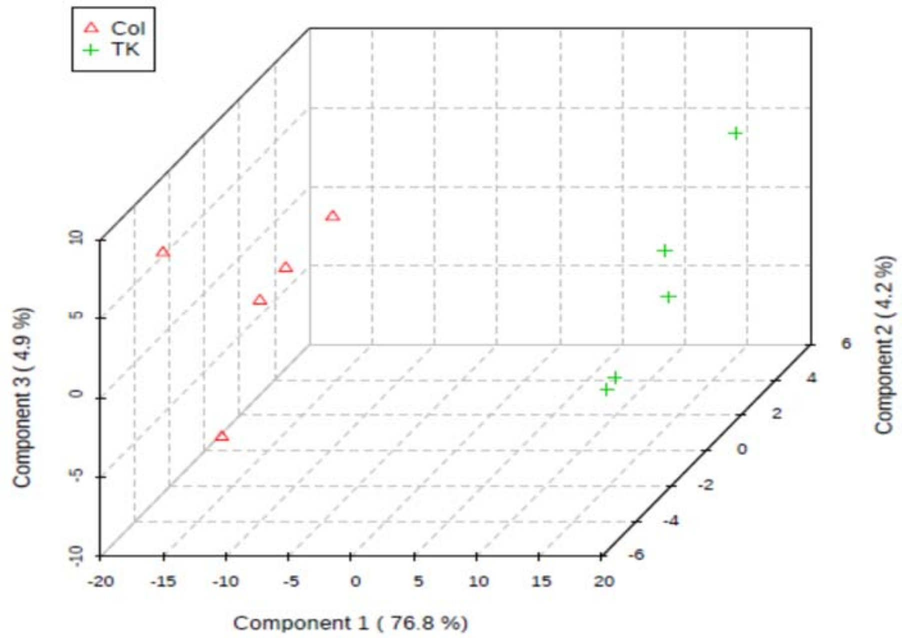

Figure A3.2: 3D score plot between the selected PCs

Appendix 4: The protocol of the bioequivalence study between Mazit 250 mg (test product) and Zithromax™ 250 mg in healthy subjects

Bioequivalence study of Neopharma, UAE Mazit capsules 250 mg (250 mg azithromycin per capsule) relative to Zithromax™ manufactured by Pfizer Italia S.R.L., Italy under authority of Pfizer Inc., USA. (250 mg azithromycin per capsule), after oral administration of 500 mg to healthy adults under fasting conditions

International Pharmaceutical Research Centre (IPRC), Jordan

Sponsor: Neopharma, UAE

Drug Identification: Azithromycin

Drug Class: Macrolides Antibiotic

Protocol Code: AZI-C017

Study Code: AZI-NEO-C0409/685

Date: August 17, 2009

Study Personnel:

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Table of Contents

International Pharmaceutical Research Centre (IPRC)
IPRC Study Code: AZI-NEO-C0409/685

Study Synopsis

Sponsor: Neopharma
Generic name: Azithromycin
Test product: Mazit capsules 250 mg
Reference product: Zithromax™
Study title: Bioequivalence study of Neopharma, UAE Mazit capsules 250 mg (250 mg azithromycin per capsule) relative to Zithromax™ manufactured by Pfizer Italia S.R.L., Italy under authority of Pfizer Inc., USA. (250 mg azithromycin per capsule), after oral administration of 500 mg to healthy adults under fasting conditions
Objectives: To investigate the bioequivalence of Mazit capsules 250 mg, (250 mg azithromycin per capsule) from (Neopharma, UAE) (Test product) relative to Zithromax™ (250 mg azithromycin per capsule), manufactured by (Pfizer Italia S.R.L, Italy), under authority of Pfizer Inc., USA., (Reference product) after a single oral dose administration of 500 mg of each to healthy adults under fasting conditions.
Dosage regimen: Treatment A (Test product): Single-dose, two capsules of Mazit Capsules 250 mg (250 mg azithromycin per capsule)
Batch No. AZA8002,
Exp. Date: 09/10
Treatment B (Reference product): Single-dose, two capsules of Zithromax™ (250 mg azithromycin per capsule)
Batch No. 86408002,
Exp. Date: 03/13
Clinical laboratories: IPRC clinical laboratories.
Study subjects: The study protocol called for 24 healthy volunteers.
Study periods: Screening: 16/04/2009, Enrolment: 22/04/2009
Period 1: 23/04/2009, Period 2: 14/05/2009
First Sample: 23/04/2009, Last sample: 17/05/2009

1. Introduction

This study was performed to investigate the bioequivalence of azithromycin between test product Mazit capsules 250 mg (250 mg azithromycin per capsule; Neopharma, UAE), and reference product Zithromax™ (250 mg azithromycin per capsule; Pfizer Inc., USA). The study protocol called for 24 healthy volunteers plus 1-4 alternates. The subjects received two capsules of Mazit capsules 250 mg (250 mg azithromycin per capsule) and two capsules of Zithromax™ (250 mg azithromycin per capsule) in a randomised fashion with a washout period of 21 days. 28 healthy volunteers enrolled and completed the crossover. The bioanalysis of clinical plasma samples was accomplished by LC/MS/MS detector which was developed and validated in accordance with international guidelines at the IPRC. Pharmacokinetic parameters, determined by standard non-compartmental methods, and ANOVA statistics were calculated using Minitab 17 Statistical Software. The 90% confidence intervals for the ratio (or difference) between the test and reference product pharmacokinetic parameters of C_{max} and truncated AUC_{0-72} were calculated.

In conclusion, the study demonstrated that the test product, Mazit capsules 250 mg (Neopharma, UAE), 250 mg azithromycin per capsule, is bioequivalent to the reference product, Zithromax™ (Pfizer Inc., USA) 250 mg azithromycin per capsule, following an oral dose of two capsules (500 mg azithromycin).

This report is issued in consensus with the ICH guidelines concerning the structure and content of the clinical study reports adopted by EMEA.

1.1. Chemistry

Azithromycin an azalide, a subclass of macrolide antibiotics, for oral administration.

Azithromycin has the chemical name (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-13-[(2,6-dideoxy-3-C-methyl-3-O-methyl- α-L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)- β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one. Azithromycin is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring. Its molecular formula is $C_{38}H_{72}N_{20}$ 12, and its molecular weight is 749.00.

Azithromycin, as the dihydrate, is a white crystalline powder with a molecular formula of $C_{38}H_{72}N_2O_{12}{\bullet}2H_2O$ and a molecular weight of 785.0.

Azithromycin is supplied for oral administration as film-coated, modified capsular shaped tablets containing azithromycin dihydrate equivalent to either 250 mg or 500 mg azithromycin.

1.2. Pharmacology

Azithromycin acts by binding to the 50S ribosomal subunit of susceptible microorganisms and, thus, interfering with microbial protein synthesis. Nucleic acid synthesis is not affected.

1.3. Pharmacokinetics

Following oral administration of a single 500 mg dose (two 250 mg tablets) to 36 fasted healthy male volunteers, the mean (SD) pharmacokinetic parameters were AUC_{0-72} = 4.3 (1.2) µg·h/mL; C_{max} = 0.5 (0.2) µg/mL; T_{max}= 2.2 (0.9) hours.

With a regimen of 500 mg (two 250 mg capsules) on day 1, followed by 250 mg daily (one 250 mg capsule) on days 2 through 5, the pharmacokinetic parameters of azithromycin in plasma in healthy young adults (18-40 years of age) are portrayed in the chart below. C_{min} and C_{max} remained essentially unchanged from day 2 through day 5 of therapy.

International Pharmaceutical Research Centre (IPRC)
IPRC Study Code: AZI-NEO-C0409/685

In a two-way crossover study, 12 adult healthy volunteers (6 males, 6 females) received 1,500 mg of azithromycin administered in single daily doses over either 5 days (two 250 mg tablets on day 1, followed by one 250 mg tablet on days 2-5) or 3 days (500 mg per day for days 1-3). Due to limited serum samples on day 2 (3-day regimen) and days 2-4 (5-day regimen), the serum concentration-time profile of each subject was fit to a 3-compartment model and the $AUC_{0-\infty}$ for the fitted concentration profile was comparable between the 5-day and 3-day regimens.

Median azithromycin exposure $AUC_{0-\infty}$ in mononuclear (MN) and polymorphonuclear (PMN) leukocytes following either the 5-day or 3-day regimen was more than a 1000-fold and 800-fold greater than in serum, respectively. Administration of the same total dose with either the 5-day or 3- day regimen may be expected to provide comparable concentrations of azithromycin within MN and PMN leukocytes. Two azithromycin 250 mg tablets are bioequivalent to a single 500 mg tablet.

1.4. Indications

Azithromycin is indicated for the treatment of patients with mild to moderate infections caused by susceptible strains of the designated microorganisms in the specific conditions listed below.

Acute bacterial exacerbations of chronic obstructive pulmonary disease due to Haemophilus influenzae, Moraxella catarrhalis or Streptococcus pneumoniae.

Acute bacterial sinusitis due to *Haemophilus injluenzae, Moraxella catarrhalis* or *Streptococcus pneumoniae.*

Community-acquired pneumonia due to *Chlamydia pneumoniae, Haemophilus injluenzae, Mycoplasma pneumoniae* or *Streptococcus pneumoniae* in patients appropriate for oral therapy.

Pharyngitis/tonsillitis caused by *Streptococcus pyogenes* as an alternative to first-line therapy in individuals who cannot use first-line therapy.

Uncomplicated skin and skin structure infections due to *Staphylococcus aureus*, *Streptococcus pyogenes*, or Streptococcus *agalactiae*. Abscesses usually require surgical drainage.

Urethritis and cervicitis due to *Chlamydia trachomatis* or *Neisseria gonorrhoeae*.

Genital ulcer disease in men due to *Haemophilus ducreyi* (chancroid). Due to the small number of women included in clinical trials, the efficacy of azithromycin in the treatment of chancroid in women has not been established.

Azithromycin, at the recommended dose, should not be relied upon to treat syphilis. Antimicrobial agents used in high doses for short periods of time to treat non-gonococcal urethritis may mask or delay the symptoms of incubating syphilis. All patients with sexually-transmitted urethritis or cervicitis should have a serologic test for syphilis and appropriate cultures for gonorrhea performed at the time of diagnosis. Appropriate antimicrobial therapy and follow-up tests for these diseases should be initiated if infection is confirmed.

Appropriate culture and susceptibility tests should be performed before treatment to determine the causative organism and its susceptibility to azithromycin. Therapy with azithromycin may be initiated before results of these tests are known; once the results become available, antimicrobial therapy should be adjusted accordingly.

To reduce the development of drug-resistant bacteria and maintain the effectiveness of azithromycin and other antibacterial drugs, azithromycin should be used only to treat or prevent infections that are proven or strongly suspected to be caused by susceptible bacteria. When culture and susceptibility information are available, they should be considered in selecting or modifying antibacterial therapy. In the absence of such data, local epidemiology and susceptibility patterns may contribute to the empiric selection of therapy.

1.5. Adverse events

Gastrointestinal tract: diarrhoea, loose stools, abdominal complaints pain, spasm, flatulence, nausea and vomiting. In patients with severe and persistent diarrhoea, the possibility of life-threatening pseudomembraneous colitis should be borne in mind.

Hepatobiliary system: A reversible increase in liver enzymes (transminases , alkaline phosphatase) and in serum bilirubin were rarely observed.

Blood and blood corpuscles: neutropenia were observed in individual cases.

Hypersensitivity reactions: are rare during treatment and include reactions of skin and mucosa such as reddening with or without pruritus. Reversible local swellings of skin, mucosa or joints (angioedema) and acute allergic general reactions (anaphylaxis) have been reported in rare cases.

Miscellaneous: superinfection with non-susceptible organisms, including fungi.

1.6. Drug interactions

Co-administration of nelfinavir at steady-state with a single oral dose of azithromycin resulted in increased azithromycin serum concentrations. Although a dose adjustment of azithromycin is not recommended when administered in combination with nelfinavir, close monitoring for known side effects of azithromycin, such as liver enzyme abnormalities and hearing impairment, is warranted.

Azithromycin did not affect the prothrombin time response to a single dose of warfarin. However, prudent medical practice dictates careful monitoring of prothrombin time in all patients treated with azithromycin and warfarin concomitantly. Concurrent use of macrolides and warfarin in clinical practice has been associated with increased anticoagulant effects.

Drug interaction studies were performed with azithromycin and other drugs likely to be co-administered. When used in therapeutic doses, azithromycin had a modest effect on the pharmacokinetics of atorvastatin, carbamazepine, cetirizine, didanosine, efavirenz, fluconazole, indinavir, midazolam, rifabutin, sildenafil, theophylline (intravenous and oral), triazolam, trimethoprim/sulfamethoxazole or zidovudine. Co-administration with efavirenz, or fluconazole had a modest effect on the pharmacokinetics of azithromycin. No dosage adjustment of either drug is recommended when azithromycin is co-administered with any of the above agents.

Interactions with the drugs listed below have not been reported in clinical trials with azithromycin; however, no specific drug interaction studies have been performed to evaluate potential drug-drug interaction. Nonetheless, they have been observed with macrolide products. Until further data are developed regarding drug interactions when azithromycin and these drugs are used concomitantly, careful monitoring of patients is advised:

Digoxin-elevated digoxin concentrations.

Ergotamine or dihydroergotamine - acute ergot toxicity characterized by severe peripheral vasospasm and dysesthesia.

Terfenadine, cyclosporine, hexobarbital and phenytoin concentrations

1.7. Warnings

Serious allergic reactions, including angioedema, anaphylaxis, and dermatologic reactions including Stevens Johnson Syndrome and toxic epidermal necrolysis have been reported rarely in patients on azithromycin therapy. Although rare, fatalities have been reported. Despite initially successful symptomatic treatment of the allergic symptoms, when symptomatic therapy was discontinued, the allergic symptoms recurred soon thereafter in some patients without further azithromycin exposure. These patients required prolonged periods of observation and symptomatic treatment. The relationship of these episodes to the long tissue half-life of azithromycin and subsequent prolonged exposure to antigen is unknown at present.

If an allergic reaction occurs, the drug should be discontinued and appropriate therapy should be instituted. Physicians should be aware that reappearance of the allergic symptoms may occur when symptomatic therapy is discontinued.

In the treatment of pneumonia, azithromycin has only been shown to be safe and effective in the treatment of community-acquired pneumonia due to *Chlamydia pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae or Streptococcus pneumoniae* in patients appropriate for oral therapy. Azithromycin should not be used in patients with pneumonia who are judged to be inappropriate for oral therapy because of moderate to severe illness or risk factors such as any of the following: patients with cystic fibrosis, patients with nosocomially acquired infections, patients with known or suspected bacteremia, patients requiring hospitalization, elderly or debilitated patients, or patients with significant underlying health problems that may compromise their ability to respond to their illness (including immunodeficiency or functional asplenia).

Clostridium difficile associated diarrhea (CDAD) has been reported with use of nearly all antibacterial agents, including azithromycin, and may range in severity from mild diarrhea to fatal colitis. Treatment with antibacterial agents alters the normal flora of the colon leading to overgrowth of *C.difficile.*

C. difficile produces toxins A and B which contribute to the development of CDAD. Hypertoxin producing strains of *C. difficile* cause increased morbidity and mortality, as these infections can be refractory to antimicrobial therapy and may require colectomy. CDAD must be considered in all patients who present with diarrhea following antibiotic use. Careful medical history is necessary since CDAD has been reported to occur over two months after the administration of antibacterial agents.

If CDAD is suspected or confirmed, ongoing antibiotic use not directed against *C. difficile* may need to be discontinued. Appropriate fluid and electrolyte management, protein supplementation, antibiotic treatment of *C. difficile,* and surgical evaluation should be instituted as clinically indicated.

1.8. Precautions

General: Because azithromycin is principally eliminated via the liver, caution should be exercised when azithromycin is administered to patients with impaired hepatic function. Due to the limited data in subjects with GFR < 10 mL/min, caution should be exercised when prescribing azithromycin in these patients.

Prolonged cardiac repolarization and QT interval, imparting a risk of developing cardiac arrhythmia and *torsades de pointes,* have been seen in treatment with other macrolides. A similar effect with azithromycin cannot be completely ruled out in patients at increased risk for prolonged cardiac repolarization.

Prescribing azithromycin in the absence of a proven or strongly suspected bacterial infection or a prophylactic indication is unlikely to provide benefit to the patient and increases the risk of the development of drug-resistant bacteria.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term studies in animals have not been performed to evaluate carcinogenic potential. Azithromycin has shown no mutagenic potential in standard laboratory tests: mouse lymphoma assay, human lymphocyte clastogenic assay, and mouse bone marrow clastogenic assay. No evidence of impaired fertility due to azithromycin was found.

Pregnancy: Teratogenic Effects. Pregnancy Category B: Reproduction studies have been performed in rats and mice at doses up to moderately maternally toxic dose concentrations (i.e., 200 mg/kg/day). These doses, based on a mg/m^2 basis, are estimated to be 4 and 2 times, respectively, the human daily dose of 500 mg. In the animal studies, no evidence of harm to the fetus due to azithromycin was found. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, azithromycin should be used during pregnancy only if clearly needed.

Nursing Mothers: It is not known whether azithromycin is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when azithromycin is administered to a nursing woman.

1.9. Contraindications

Azithromycin is contraindicated in patients with known hypersensitivity to azithromycin, erythromycin, any macrolide or ketolide antibiotic.

2. Selection of study population

For participation in the study, subjects had to meet the selection criteria outlined in the study protocol. Volunteers were informed, by IPRC representative, about the aim of the study and any potential risk associated with the study. Volunteers signed a written Informed Consent statement after which they were run in the study, and they were free to withdraw at any time during the course of the study.

2.1. Study subjects demography

The following demographic data for each subject were obtained:

- Name, sex, age, height, weight, date of birth, race, medical history, vital signs and drug of abuse tests, caffeine, alcohol and tobacco intake.
- Complete physical examination and ECG
- Urine analysis and Blood (haematology, biochemistry and serology).

2.2. Subjects identification

On screening subjects were identified solely by their initials. On admission for period one participating subjects were assigned numbers in sequential order. The subjects retained their numbers for the duration of the study. For subsequent data processing and reporting, subjects were identified only by using the numbers they were assigned and their initials.

2.3. Case Report Form Note

All data of the clinical part of the study was documented in case .report forms (CRFs) by the staff of the IPRC. The Principal Investigator checked correct completion of the case report forms. IPRC performed quality assurance of case report forms' data entry by comparison with source records.

2.4. Confinement

According to the study protocol in each study period the subjects were admitted to the study site in the evening before study drug administration on study day 1 of each study period and confined until the 24-hour blood sample was collected and returned to donate the rest of samples.

2.5. Study drug administration

On study day 1 of each study period, the study drugs were administered according to a randomization plan. The administration of the study drugs was documented in the drug administration form.

Study drugs were administered by the clinical staff of IPRC as follows:

Treatment A: Two capsules of *Mazit capsules 250 mg,* test product, 250 mg azithromycin per capsule was given with 240 ml of water. Water was at room temperature and was measured with a 250 ml cylinder.

Treatment B: Two capsules of *Zithromax™,* reference product, 250 mg azithromycin per capsule was given with 240 ml of water. Water was at room temperature and was measured with a 250 ml cylinder.

2.6. Blood samples

In the morning of study day 1 of each study period and before study's drugs administration, a cannula was inserted into the subject's forearm vein and remained there until the 24-hour blood sample was collected and then the subject returned to donate the rest of samples.

The volume of blood taken for determination of azithromycin in plasma was 8 mL per sample. The following blood samples for the analysis of azithromycin in plasma were collected immediately before (2 x 8 mL) at 0.00 (pre- dose) and at 0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00 hours, (1 x 8 mL) after administration of study drugs. The number of blood collections for drug analysis was 22 samples in each study period.

3. Saftey evaluation

Adverse events encountered during the study were minimal. The study outcome will help ensure safe and clinically reliable management of acute bacterial exacerbations of chronic obstructive pulmonary disease, acute bacterial sinusitis, community-acquired pneumonia, pharyngitis/tonsillitis, uncomplicated skin and skin structure infections, urethritis and cervicitis, genital ulcer disease therefore, benefiting society by lowering treatment costs. The drug is a prescribed medication, we therefore conclude that in view of the small risks involved, it was significant to perform this study.

3.1. Adverse events during the study

The study's subjects were asked to inform the clinical staff of occurrence of any AE immediately once experienced. Furthermore, the clinical staff was instructed to check on the subjects for the occurrence of any AE at specified time intervals (before dosing, 1.00, 2.00, 3.00, 4.00, 7.00, 9.00 and 12.00 hours from study drugs administration) and to notify immediately the study physician. The study physician monitored closely the subjects for AE and took all necessary actions that he saw best in the subject's interest. None of the subjects dropped out from the study because of adverse events.

3.2. Clinical laboratory evaluation

Medical histories and the laboratory tests of haematology, serology, biochemistry and urinalysis. Were all performed for each subject on screening examination. Only medically healthy subjects with clinically normal laboratory profiles were enrolled in the study.

3.3. Vital signs, physical assesment and other clinical oservation

Each subject received a thorough physical assessment, vital signs evaluation (blood pressure, pulse, respiratory rate and temperature) and ECG on screening examination. The subjects received the same physical assessment and vital signs evaluation and ECG plus liver functions test on follow up examination, which was within at least 24 hours from collecting the last sample in period 2.

4. Ethics considerations

4.1. Basic principle

This research will be carried out in accordance with conditions stipulated by international clinical research guidelines and the principles enunciated in the Declaration of Helsinki resolved in Helsinki in 1964 and amended in Scotland, 2000; note added in Washington, 2002, note added in Tokyo 2004 also an updated was done in Seoul 2008 and the ICH harmonised tripartite guideline regarding Good Clinical Practice (GCP) adopted by the European Agency for the Evaluation of Medicinal Products. In addition, all local regulatory requirements will be adhered to, in particular those which afford greater protection to the safety of the study participants.

4.2. Institutional Review Board (IRB)

The Institutional Review Board of the IPRC will review the protocol and the study will not start until the Board has approved the protocol or a modification thereof. The Board is constituted and operates in accordance with the principles and requirements described in the Guidelines on Research Involving Human Subjects. All amendments to the study protocol are to be sent to the IRB for approval.

4.3. Informed consent

The purpose of the study, the procedures to be carried out and the potential hazards will be describe to the subjects in non-technical terms. Subjects will be required to read, sign and date a consent for summarising the discussion prior to enrolment and will be assured that they may withdraw from the study at any time without jeopardising their medical care. Each subject will be given a copy of the consent form.

4.4. Subject confidentiality

All communications and documents relevant to subjects in the study will identify each subject only by the subjects' initials or by the subjects' study numbers.

4.5. Subjects indemnity

The sponsor will provide the insurance with the legal and financial coverage against claims arising from the study medications. This indemnity will be mediated by the International Pharmaceutical Research Centre, before conducting the study. The cost of treatment of study subjects in the event of study-related injuries are settled on and stated in the contract of the insurance company. Each subject will be informed about his indemnity in the Informed Consent Form (ICF), with clear and complete declaration for the undertaken responsibility.

In the event of a study-related injury, the clinical staff of the IPRC will provide immediate medical treatment, free of charge and provide subsequent referrals to the appropriate health care facilities. The IPRC, on behalf of the sponsor, will be responsible for the financial coverage of all the medical expenses of injuries suffered as a result of participating in this study.

4.6. Subjects compensation

Compensation process will be defined for cases of withdrawals, completion, termination and suspension. The method and manner of compensation by which study subjects will receive their payment would be stated clearly in the Informed Consent Form (ICF).

5. Data analysis

5.1. Pharmacokinetic analysis

C_{max} and truncated AUC_{0-72} are considered the primary pharmacokinetic parameters, while t_{max} and $AUC_{0-\infty}$ are defined as secondary pharmacokinetic parameters. Pharmacokinetic parameters will be calculated as follows using the Minitab 17 computer program:

AUC_{0-72}:The area under the plasma concentration versus time curve, from time (0) to 72 hours, as calculated by the linear trapezoidal method.

$AUC_{0-\infty}$: The area under the plasma concentration versus time curve from time (0) to infinity. $AUC_{0-\infty}$ is calculated as the sum of the AUC_{0-t} plus the ratio of the last measurable plasma concentration to the elimination rate constant.

C_{max}: Maximum measured plasma concentration over the time span specified.

t_{max}: Time ofthe maximum measured plasma concentration. If the maximum value occurs at more than one time point, t_{max} is defined as the first time point with this value.

5.2. Analysis of variation

Analyses of variance will be performed on the untransformed pharmacokinetic parameters listed above. Additionally, logarithmically transformed data will be used for analysis of truncated AUC_{0-72} and C_{max}.The analysis of variance model will include sequence; subjects nested within sequence, period and drug formulation as factors, employing 5% level of significance. The significance of the sequence effect will be tested using the subjects nested within sequence, as the error term.

5.3. Confidence intervals and bioequivalence evaluation

Consistent with the two one-sided tests for bioequivalence, 90% confidence intervals for the difference between drug formulation means will be calculated for the log-transformed parameters truncated AUC_{0-72} and C_{max} for azithromvcin. The confidence intervals will be expressed as a percentage relative to the means of the reference formulation. The geometric mean values for the (test/reference) ratios of truncated AUC_{0-72} and C_{max} will be reported to define the point estimate. The confidence intervals of logarithmically transformed (test/reference) ratios for C_{max} and truncated AUC_{0-72} to be within 80.00-125.00%.

5.4. Sample size calculation

Previous studies carried concerning Azithromycin in IPRC indicated that the variability does not imply large sample size to be employed for bioequivalence determination, calculated sample size showed that a minimum of 12 subjects will be adequate. Sample size calculation is based on the power of Schuirmann's two one-sided tests procedure for interval hypotheses using the ± 20 rule for the assessment of average bioequivalence.

6. Final report, supplemtary documentation and publication policy

Final Report: All reporting will be performed by IPRC. The final report will address all aspects of the study and will include the interpretation of all relevant data and any conclusions from them. A copy from the final report shall be reserved by IPRC after the completing the study. A sample informed consent form, a sample CRF, a copy of the IRB approval and the bioanalytical method validation report will be appended to the final report. The subjects' CRFs, clinical laboratory tests; a set of representative authentic clinical sample chromatograms with their results, standard calibration curves and quality control samples shall be supplemented on request. The final report shall be signed by the IPRC Investigator(s) and submitted to the sponsor with the other study supplements.

Source Documents: Source documents include original documents, data, and records (e.g., hospital records, clinical and office charts, laboratory notes, memoranda, accountability records, recorded data from automated instruments, informed consent forms, case report forms, bioanalytical results and chromatograms, pharmacokinetic spreadsheets, copies or transcriptions certified after verification as being accurate copies, microfiches, photographic negatives, microfilms or magnetic media, x-rays, subject files, and records kept at any department involved in the trial). All source documents generated in connection with this study will be retained in the limited access file storage area of IPRC, respecting the privacy and confidentiality of all records that could identify the subjects. Direct access is allowed only for authorized people for monitoring and auditing purposes. Source documents shall be handled, stored and archived according to in-house procedures to assure for accurate reporting, interpretation and verification, under the supervision of the Quality Assurance Unit.

Publication Policy: All information concerning Mazit capsules 250 mg and the Sponsor operations. such as patent applications, formulae, manufacturing processes, basic scientific data or formulation information, supplied by Sponsor and not previously published is considered confidential by the Principal Investigator and Study Director.

International Pharmaceutical Research Centre (IPRC)
IPRC Study Code: AZI-NEO-C0409/685

References

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Appendix 5: The Research Ethics Committee approval for the bioequivalence study of Mazit 250 mg (test product) and Zithromax™ 250 mg (reference product)

المؤسسة العامة للغذاء والدواء

الرقم ....
التاريخ ....
الموافق ....

السادة المركز العالمي للأبحاث الصيدلانية

تحية طيبة وبعد،،،

إشارة إلى كتابكم رقم بلا بتاريخ 2009/4/9 بخصوص دراسة التكافؤ الحيوي التالية :

Comparative, randomized, single dose, two-way crossover open-label study to determine the bioequivalence of *Neopharma, UAE Mazit Capsules 250mg* (250mg Azithromycin / Capsule) relative to *Zithromax*™ Manufactured by Pfizer Italia S.R.L., Italy Under Authority of Pfizer INC., USA. (250mg Azithromycin / Capsule), after an oral administration of *500mg* to healthy adults under fasting conditions.
Protocol Code No.: AZI-C017

والمقدمة تحت الرقم 28/BioNR/09

وبعد الإطلاع ، وبناءا على تنسيب لجنة الدراسات الدوائية في جلستها المنعقدة بتاريخ 2009/4/14 أعلمكم بالموافقة على إجراء الدراسة بالبروتوكول أعلاه بعد تزويد قسم الدراسات الدوائية / المؤسسة العامة للغذاء والدواء بإلتزامكم بإجراء فحوصات الECG, Hematology, Biochemistry للمشاركين إذا كانت الفترة بين إجراء فحوصات الScreening وإجراء الفترة الثانية من الدراسة شهر فأكثر مع ضرورة الإلتزام بقانون إجراء الدراسات الدوائية رقم 67 لسنة 2001 والملاحق الصادرة عنه.

واقبلوا تحياتي،،،

وزير الصحة

الدكتور نايف هايل الفايز

- نسخة / قسم الدراسات الدوائية
HLCTU2/15/2009

جبل عمان – الدوار الثالث – ص.ب 811951 الرمز البريدي 11181 – هاتف : 4602000 – تلفاكس : 4618425-6-962+
Amman – Jordan Tel. 4602000 – Telefax: +962 - 6 – 4618425 P.O.Box 811951 P.C 11181 - www.jfda.jo

Translation of the Research Ethics Committee approval for the bioequivalence study

The Food and Drug Administration

Amman, Jordan

No.: 6/119
Date: 2/4/1437
Date: 23/April/2009

To the International Centre for Pharmaceutical Research

Greetings,

Reference to your letter dated on 9/April/2009 regarding the following bioequivalence study: Comparative, randomised, single dose, two-way crossover open-label study to determine the bioequivalence of Neopharma, UAE Mazit Capsules (250mg Azithromycin/ Capsules) relative to Zithromax™ Manufactured by Pfizer Italia S.R.L, Italy Under Authority of Pfizer INC., USA. (250mg Azithromycin/ Capsules), after an oral administration of 500mg to healthy adults under fasting conditions. Protocol Code No.: AZI-C017

Which is submitted under the following number: 28/BioNR/09

After reviewing and based on the recommendation of the Research Ethics Committee of pharmacological studies meeting which is held on 14/April/2009 I would like to inform you of the approval of the study protocol mentioned above, after providing the pharmaceutical department/ Food and Drug Administration your commitment to do the following screening test: ECG, Haematology, and Biochemistry to all participants in the study if the period between conducting the screening examinations and the second period of the study is one month or more, with the necessity to comply with the Law of Pharmaceutical Studies No. 67/ year 2001 and the supplements issued.

Best regards,

Minister of Health

Dr. Nayef Hayel Al-Fayez

Copy / Department of Pharmaceutical Studies

Translated By:...B. Aljohani.........

Signature: ....

Approved by: ...ESSAM...KERWASH

Signature: ....Essam...Kerwash

Professor Atholl Johnston FRCPath
Barts and The London
Queen Mary's, University of London
Charterhouse Square
London, EC1M 6BQ, UK

Appendix 6: Raw data for all subjects who participated in the bioequivalence study of Mazit 250 mg (test product) and Zithromax™ 250 mg (reference product)

Table A6.26: Schedule of vital signs, physical assessment and other clinical observations during the bioequivalence study

Study Day	Nominal Time (h) Relative to drug administration	Blood collection	Vital Signs	Hospitalization
-1	-10.00 at least	Standardized Dinner		✓
1	Pre-dose	✓	✓	✓
1	(0.00)	Study Drug Administration*		✓
1	0.33	✓		✓
1	0.66	✓		✓
1	1.00	✓	✓	✓
1	1.33	✓		✓
1	1.66	✓		✓
1	2.00	✓	✓	✓
1	2.50	✓		✓
1	3.00	✓	✓	✓
1	3.50	✓		✓
1	4.00	✓	✓	✓
		Standardized Lunch		
1	4.50	✓		✓
1	5.00	✓		✓
1	6.00	✓	✓	✓
1	8.00	✓	✓	✓
		Snack		
1	10.00	✓		✓
1	11.00		✓	✓
1	12.00	✓		✓
		Standardized dinner		
1	16.00	✓		✓
2	24.00	✓	✓	✓
3	48.00	✓		
4	72.00	✓		

* *Study drugs will be administered with 240 ml water. Fluids intake is not allowed from 1-hour prior study drug administration, until one hour after study drug administration. There after, the subjects will be allowed to drink water as desired*

Table A6.27: Subjects and reasons of withdrawal from the bioequivalence study

Withdrawn Subject	Group[1]	Reason For Withdrawal
TMM	1	Abnormal lab. results
IMA	1	Personal reason
AHO	1	Medical condition
AMO	1	Medical condition
IIM	1	Personal reason
RMD	1	Abnormal lab. results
MOI	1	Abnormal lab. results
AAM	1	Abnormal lab. results
EME	1	Abnormal lab. results
MAA	1	Abnormal lab. results
MHO	1	Personal reason
MAS	1	Personal reason
JAS	1	Personal reason
MAL	1	Personal reason
MYA	1	Personal reason
SMB	1	Personal reason
SMA	1	Personal reason
HMM	1	Personal reason
KKA	1	Personal reason
MFM	1	Personal reason

Table A6.28: Demographic data and individual values for eligible subjects included in the bioequivalence study

Subject No.	Body Height (cm)	Body Weight (kg)	Age (yr)	Race	Sex
1	165	80	31	Caucasian	Male
2	171	60	26	Caucasian	Male
3	170	66	22	Caucasian	Male
4	178	60	24	Caucasian	Male
5	188	90	32	Caucasian	Male
6	178	65	31	Caucasian	Male
7	160	65	34	Caucasian	Male
8	172	70	28	Caucasian	Male
9	187	88	30	Caucasian	Male
10	182	85	24	Caucasian	Male
11	170	65	22	Caucasian	Male
12	177	60	23	Caucasian	Male
13	162	60	37	Caucasian	Male
14	172	70	28	Caucasian	Male
15	167	70	25	Caucasian	Male
16	171	70	36	Caucasian	Male
17	181	85	24	Caucasian	Male
18	170	82	26	Caucasian	Male
19	175	60	30	Caucasian	Male
20	175	65	25	Caucasian	Male
21	176	75	20	Caucasian	Male
22	181	70	28	Caucasian	Male
23	174	80	25	Caucasian	Male
24	180	80	22	Caucasian	Male
25	175	80	27	Caucasian	Male
26	183	70	29	Caucasian	Male
27	174	65	37	Caucasian	Male
28	176	68	21	Caucasian	Male

Table A6.29: Drug administration times for all subjects in period 1 and 2

Subject No.	Period I		Period II	
	Date (DD/MM/YY)	Time (HH:MM)	Date (DD/MM/YY)	Time (HH:MM)
1	23/04/2009	8:00	14/05/2009	8:00
2	23/04/2009	8:02	14/05/2009	8:02
3	23/04/2009	8:04	14/05/2009	8:04
4	23/04/2009	8:06	14/05/2009	8:06
5	23/04/2009	8:08	14/05/2009	8:08
6	23/04/2009	8:10	14/05/2009	8:10
7	23/04/2009	8:12	14/05/2009	8:12
8	23/04/2009	8:14	14/05/2009	8:14
9	23/04/2009	8:16	14/05/2009	8:16
10	23/04/2009	8:18	14/05/2009	8:18
11	23/04/2009	8:20	14/05/2009	8:20
12	23/04/2009	8:22	14/05/2009	8:22
13	23/04/2009	8:24	14/05/2009	8:24
14	23/04/2009	8:26	14/05/2009	8:26
15	23/04/2009	8:00	14/05/2009	8:00
16	23/04/2009	8:02	14/05/2009	8:02
17	23/04/2009	8:04	14/05/2009	8:04
18	23/04/2009	8:06	14/05/2009	8:06
19	23/04/2009	8:08	14/05/2009	8:08
20	23/04/2009	8:10	14/05/2009	8:10
21	23/04/2009	8:12	14/05/2009	8:12
22	23/04/2009	8:14	14/05/2009	8:14
23	23/04/2009	8:16	14/05/2009	8:16
24	23/04/2009	8:18	14/05/2009	8:18
25	23/04/2009	8:20	14/05/2009	8:20
26	23/04/2009	8:22	14/05/2009	8:22
27	23/04/2009	8:24	14/05/2009	8:24
28	23/04/2009	8:26	14/05/2009	8:26
Minimum		***8:00***	***Minimum***	***8:00***
Maximum		***8:26***	***Maximum***	***8:26***

Table A6.30: Adverse events for all subjects during the study

Subject No.	Adverse Event(s) Occurred
1	No
2	No
3	No
4	Yes
5	No
6	No
7	Yes
8	Yes
9	Yes
10	No
11	No
12	No
13	No
14	Yes
15	No
16	No
17	No
18	No
19	No
20	No
21	No
22	No
23	No
24	No
25	No
26	No
27	No
28	No

No: Adverse event(s) did not occur.
Yes: Adverse event(s) did occur.

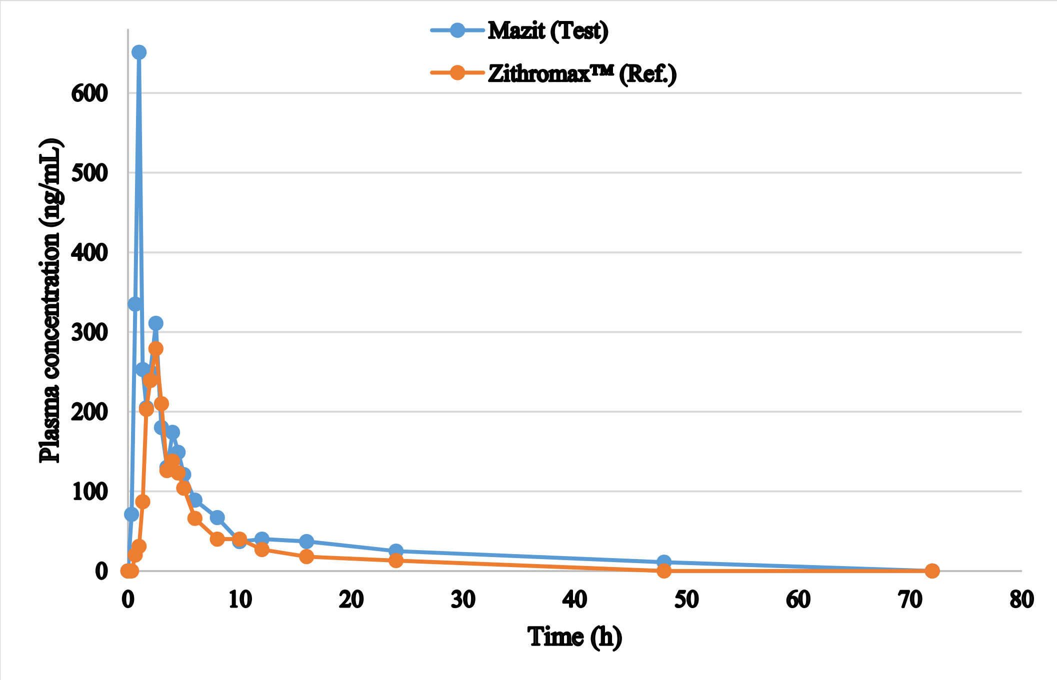

Figure A6.3: Concentration of Mazit compared to Zithromax™ (Subject 2)

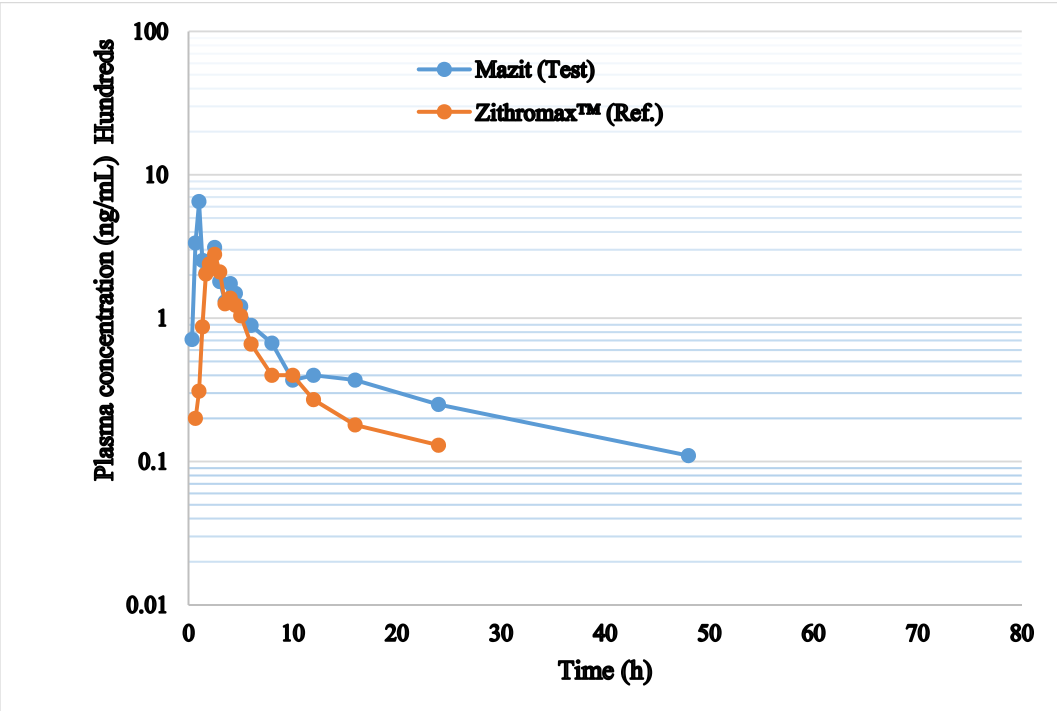

Figure A6.4: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 2)

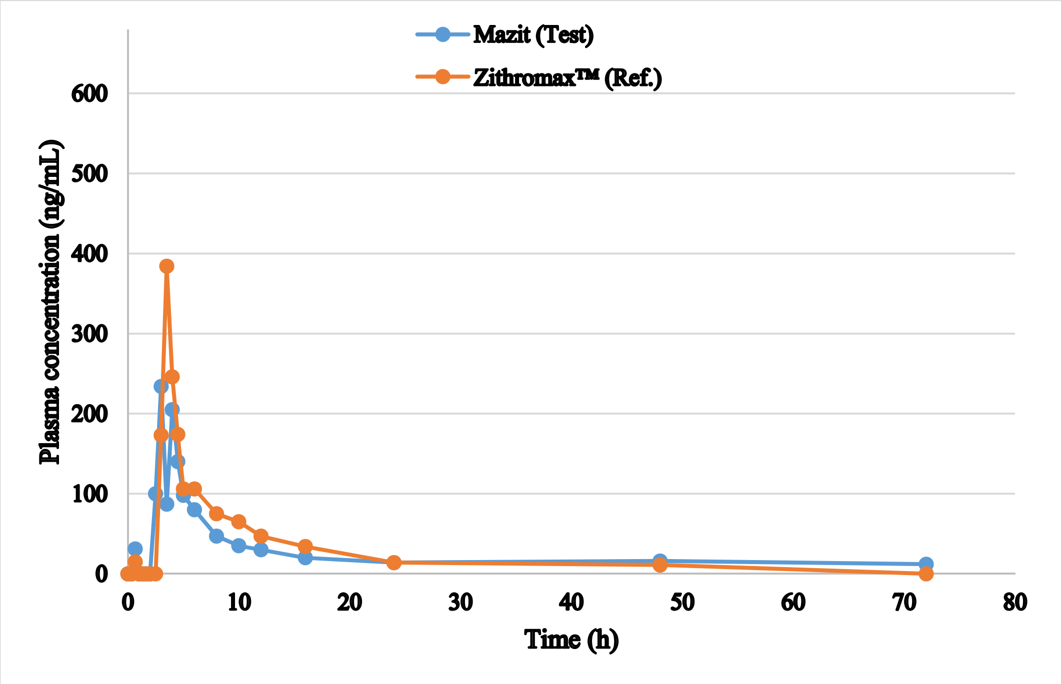

Figure A6.5: Concentration of Mazit compared to Zithromax™ (Subject 3)

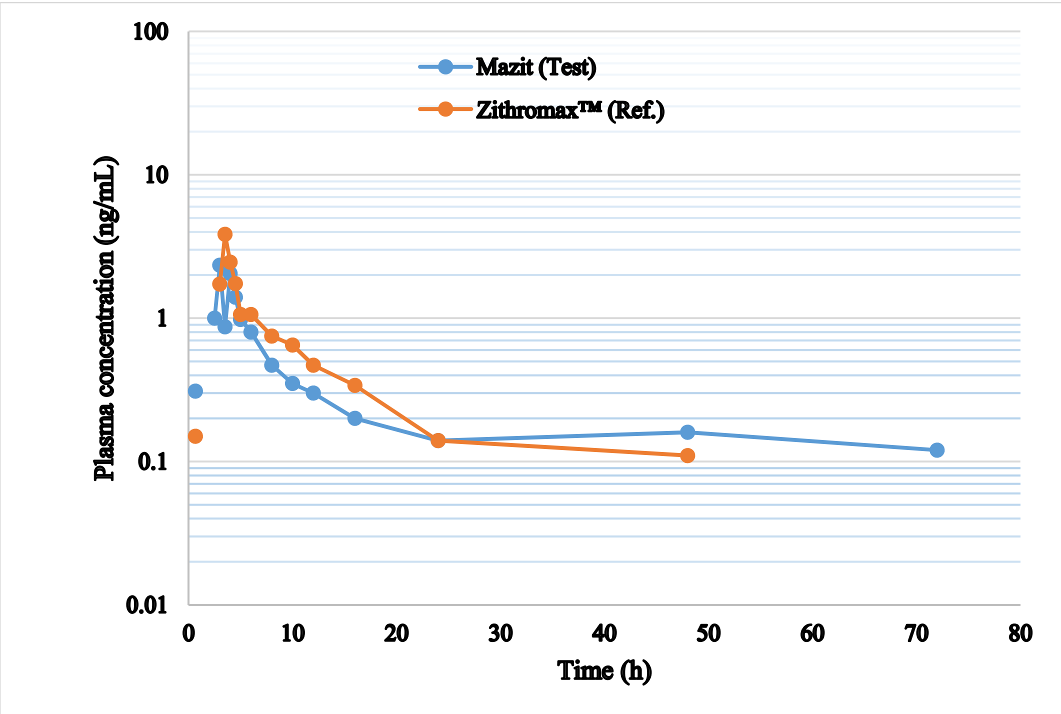

Figure A6.6: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 3)

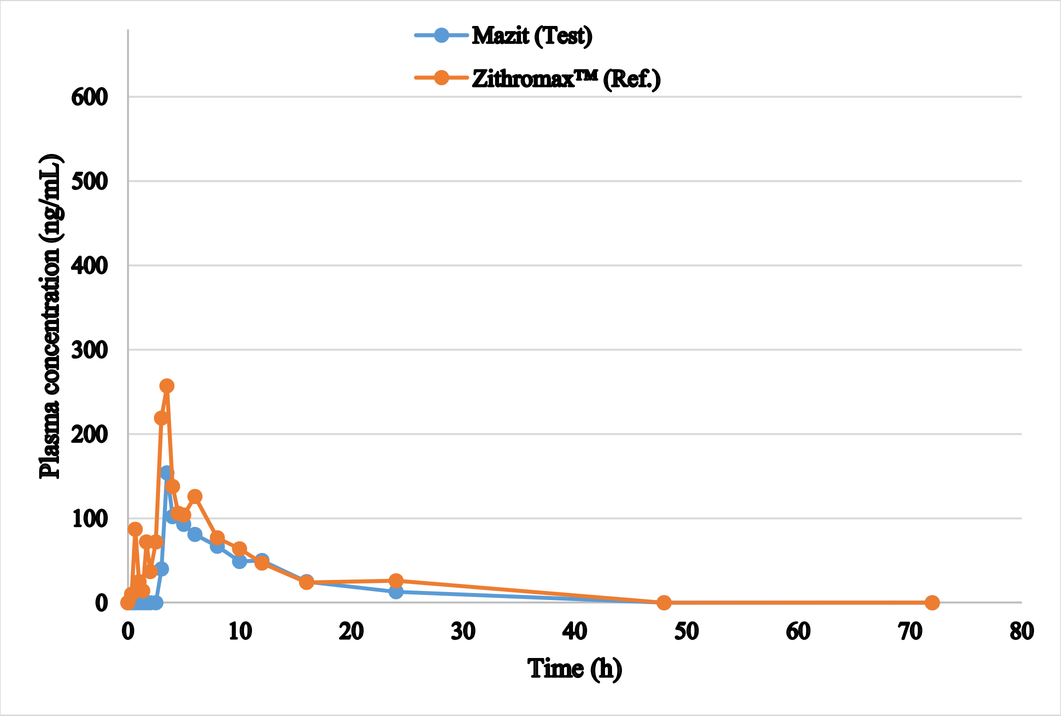

Figure A6.7: Concentration of Mazit compared to Zithromax™ (Subject 4)

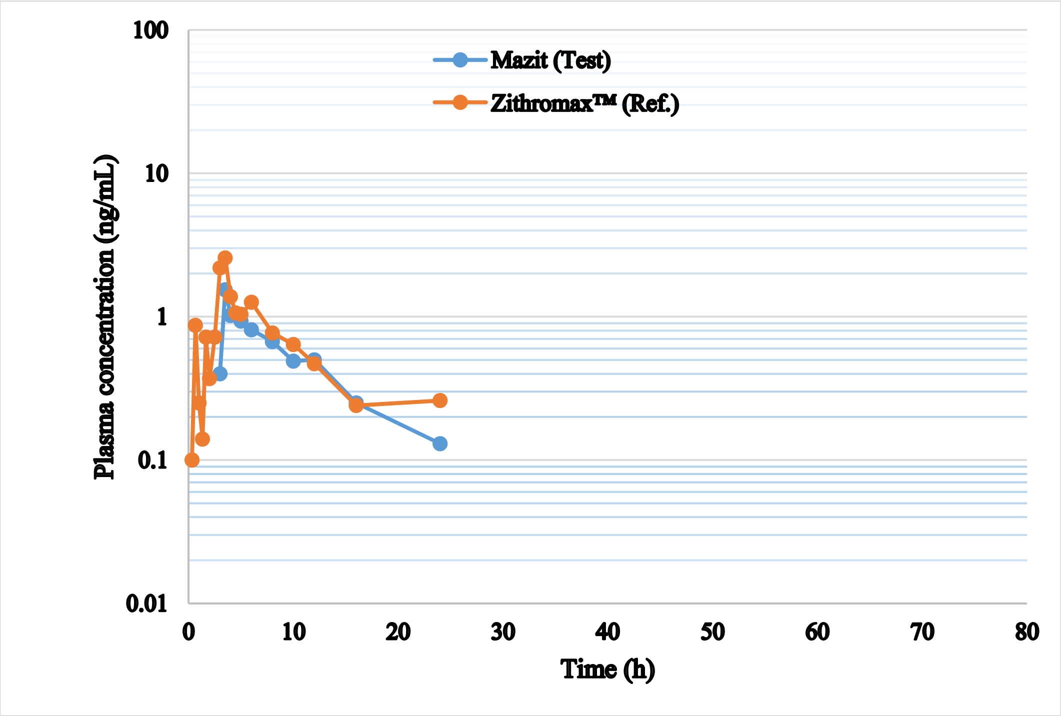

Figure A6.8: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 4)

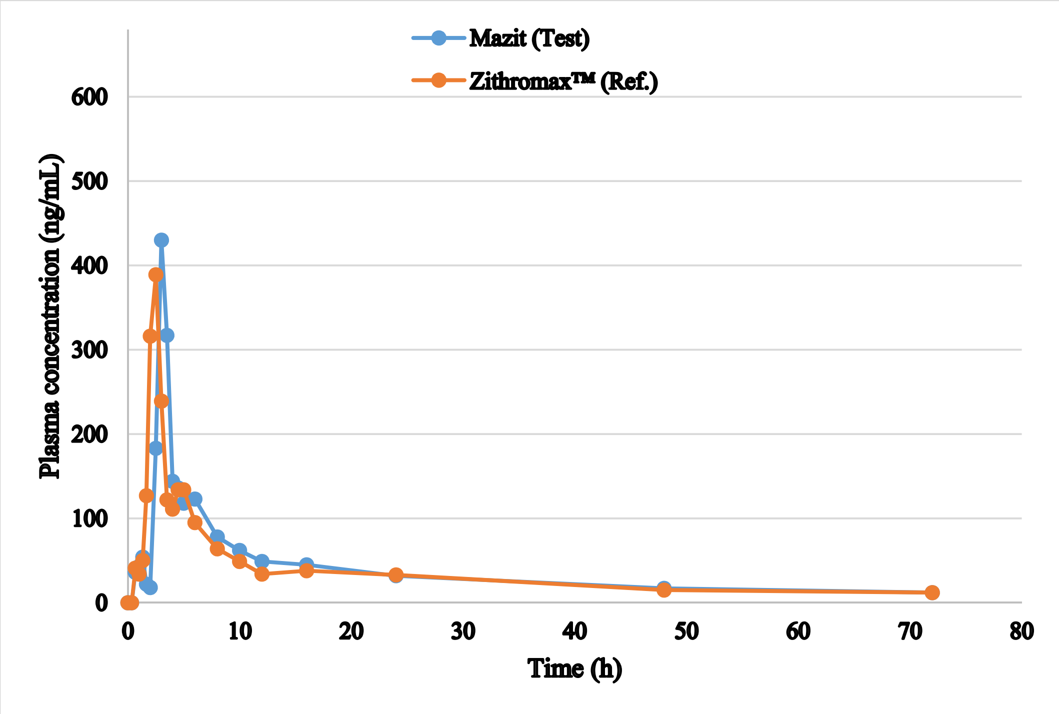

Figure A6.9: Concentration of Mazit compared to Zithromax™ (Subject 5)

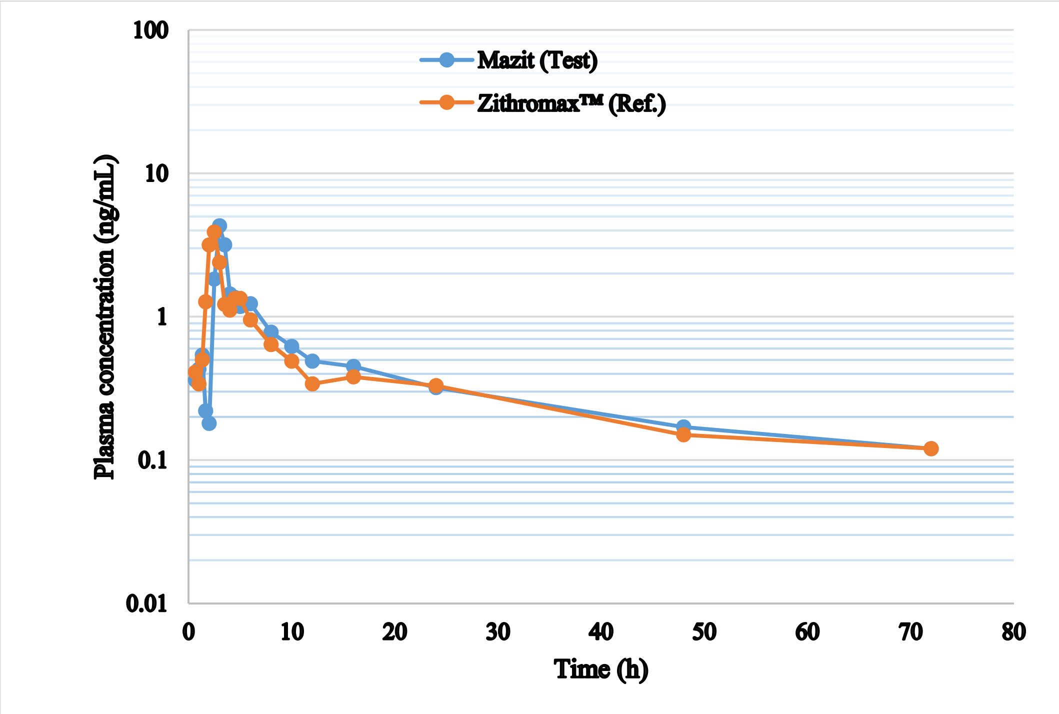

Figure A6.10: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 5)

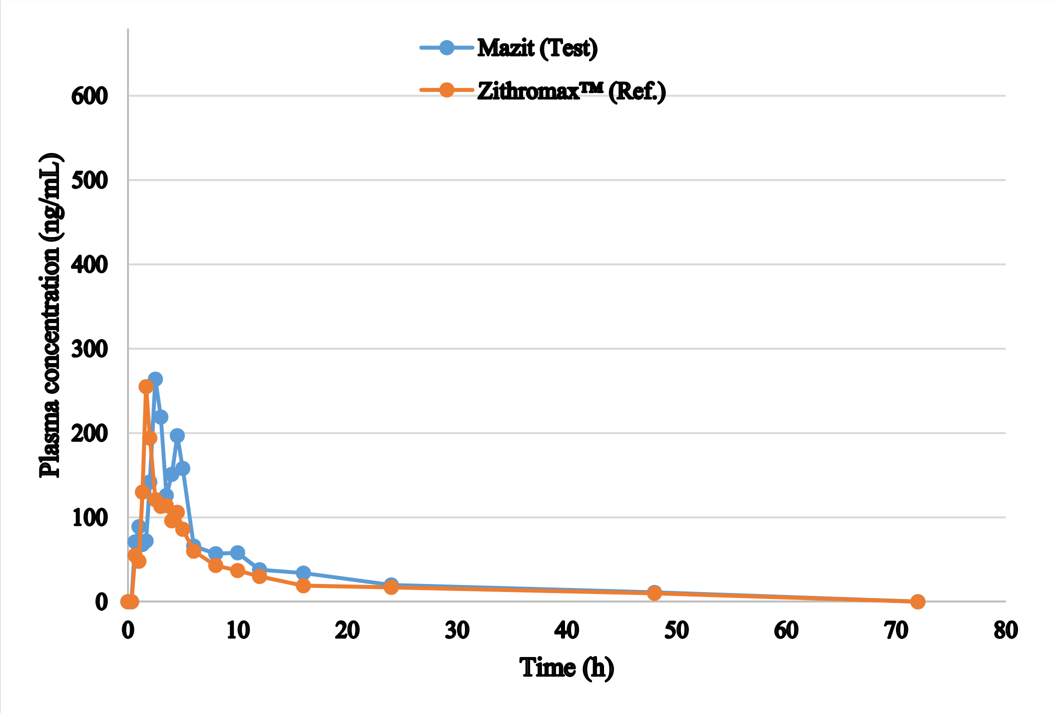

Figure A6.11: Concentration of Mazit compared to Zithromax™ (Subject 6)

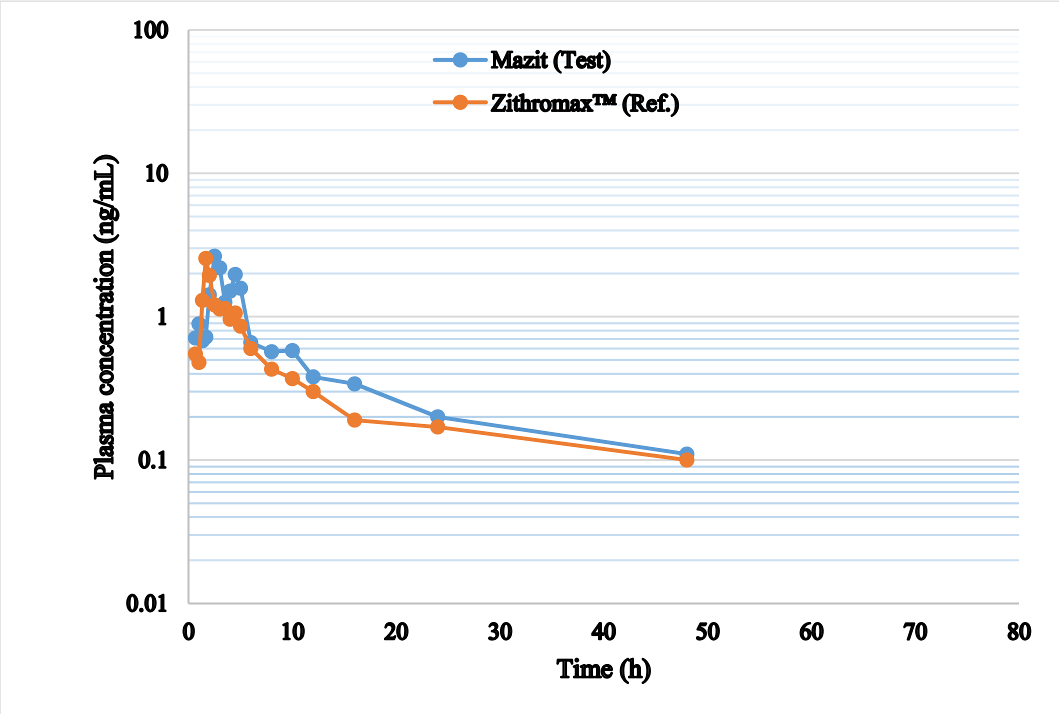

Figure A6.12: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 6)

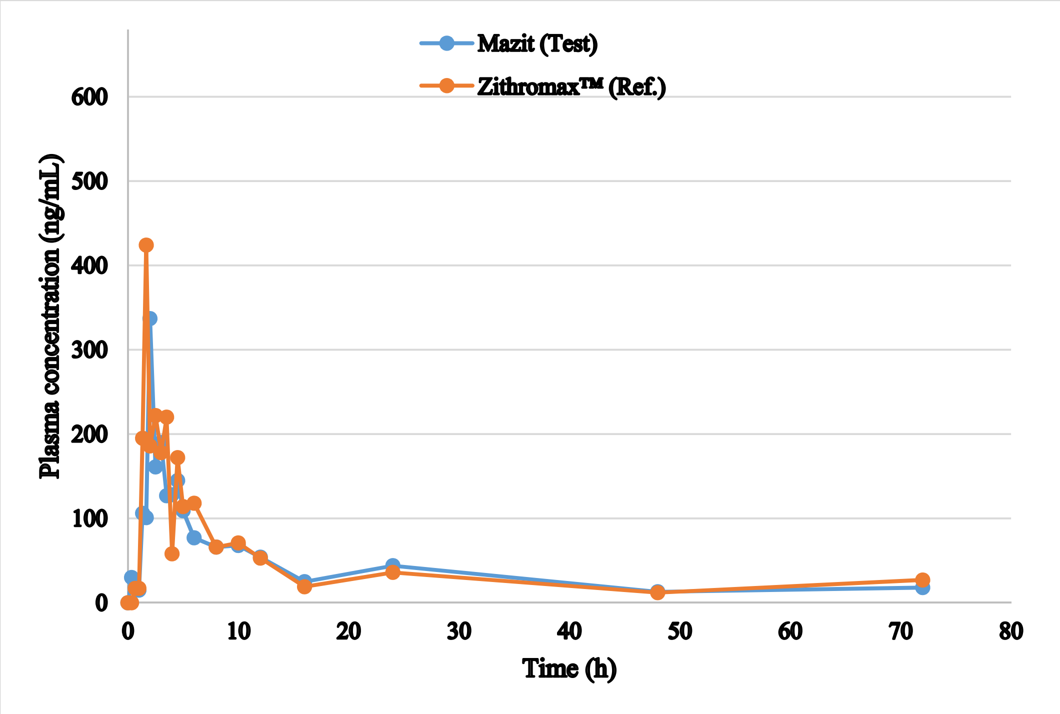

Figure A6.13: Concentration of Mazit compared to Zithromax™ (Subject 7)

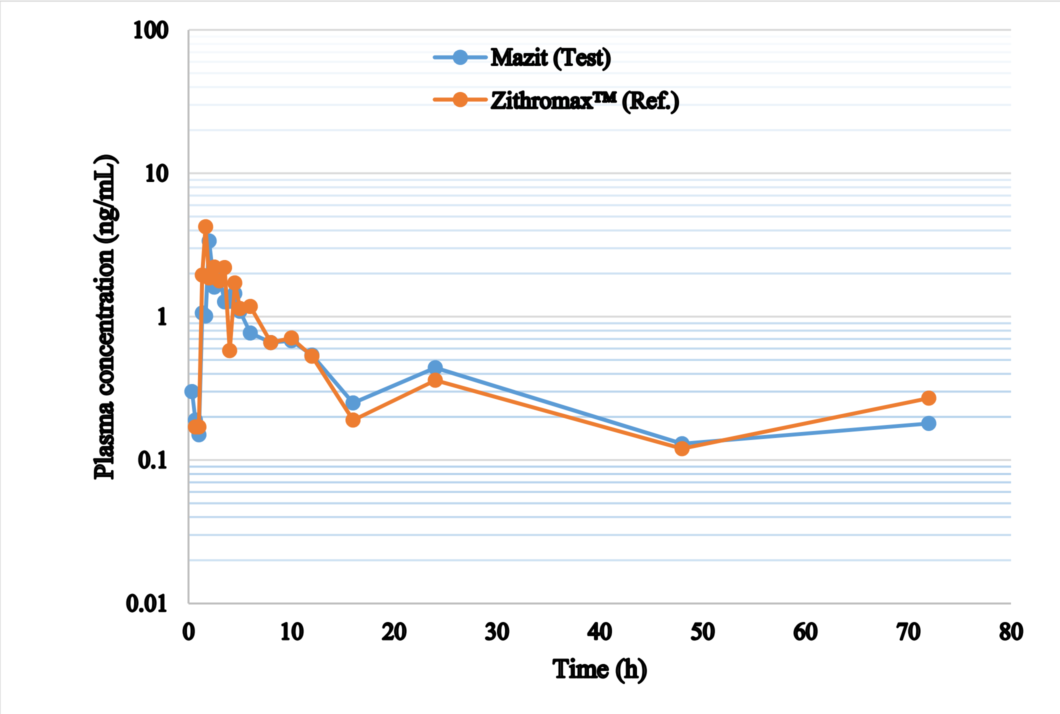

Figure A6.14: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 7)

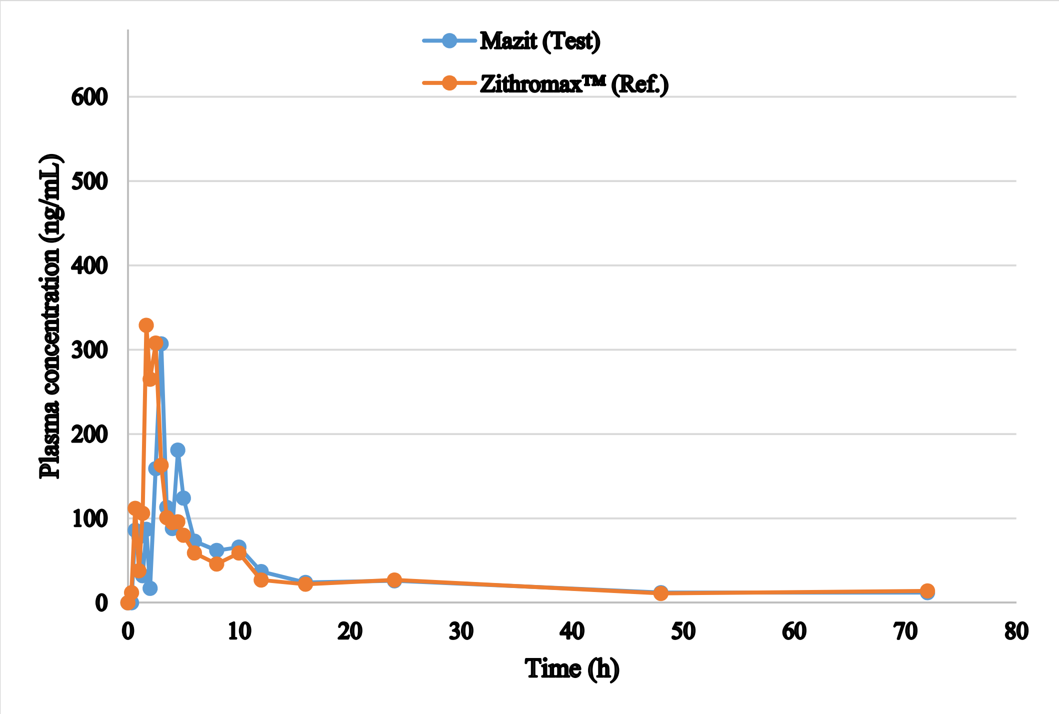

Figure A6.15: Concentration of Mazit compared to Zithromax™ (Subject 8)

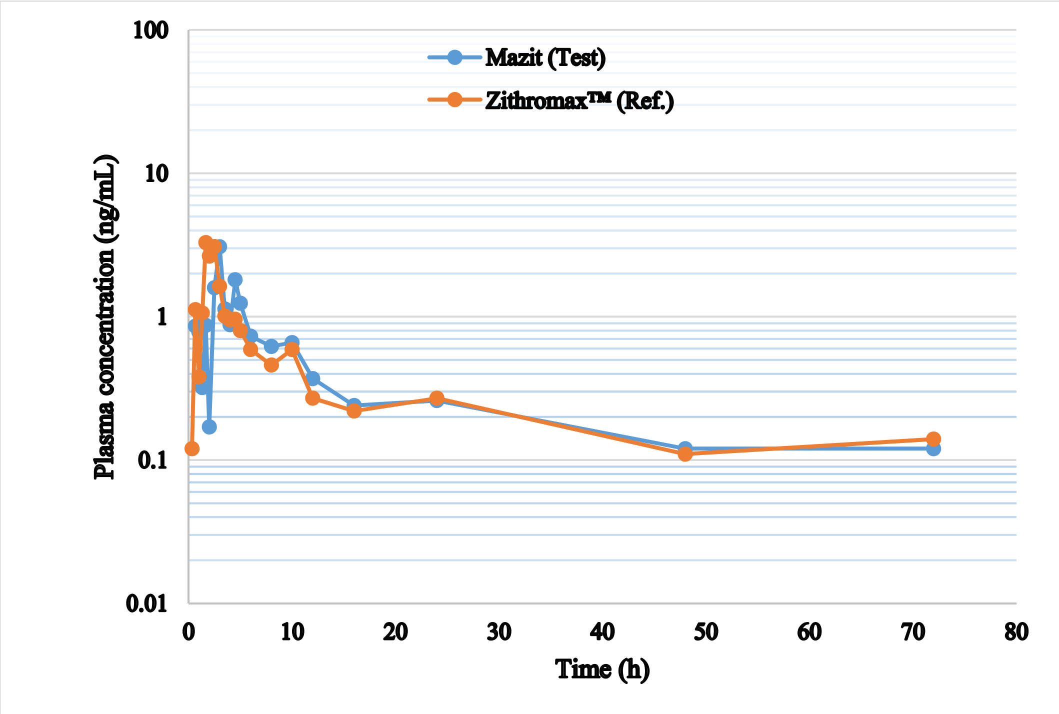

Figure A6.16: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 8)

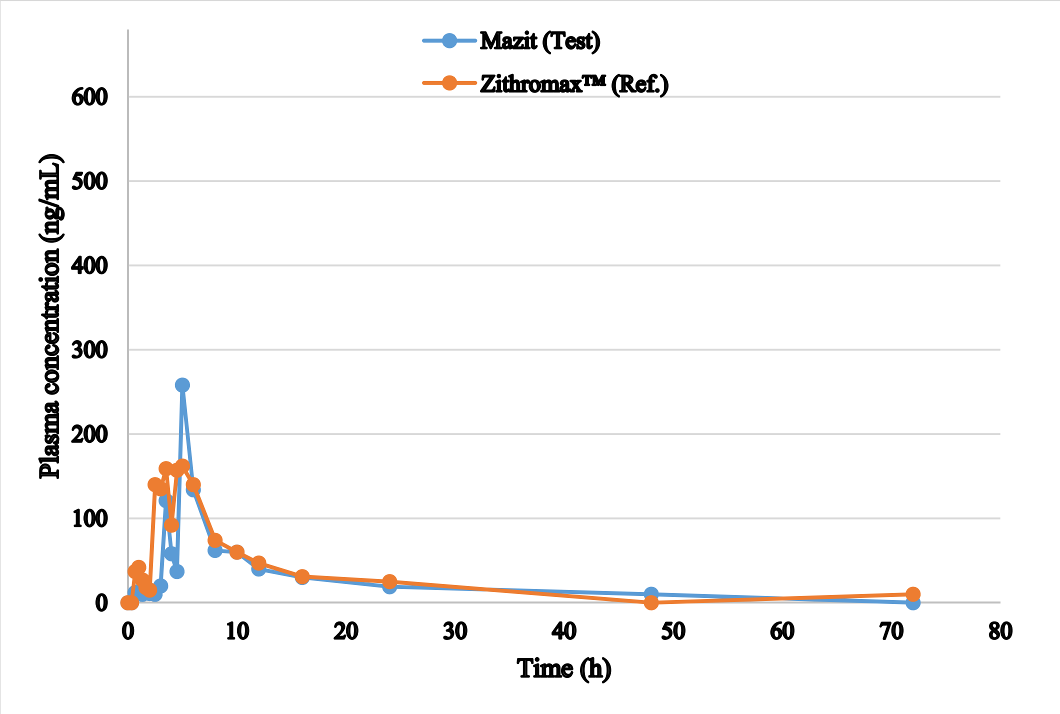

Figure A6.17: Concentration of Mazit compared to Zithromax™ (Subject 9)

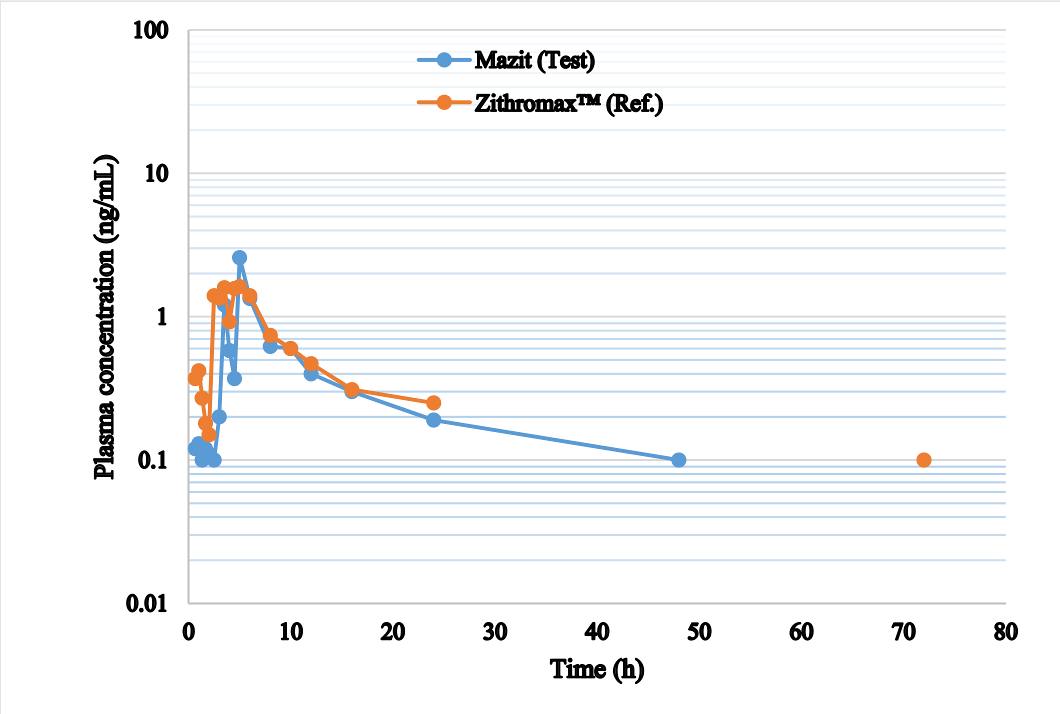

Figure A6.18: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 9)

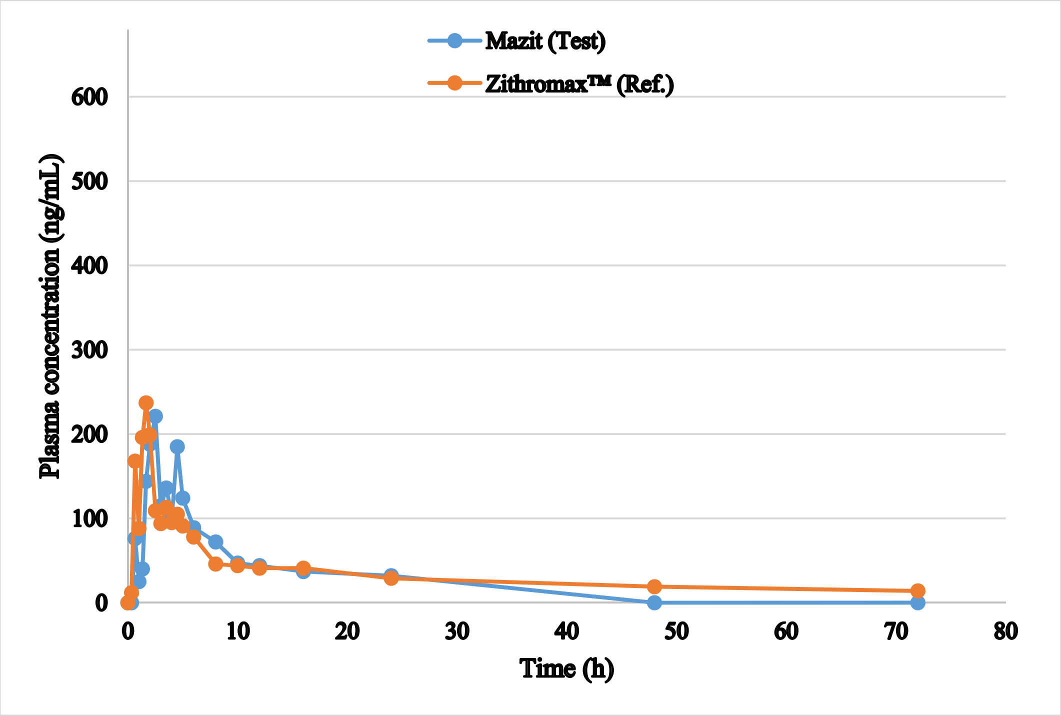

Figure A6.19: Concentration of Mazit compared to Zithromax™ (Subject 10)

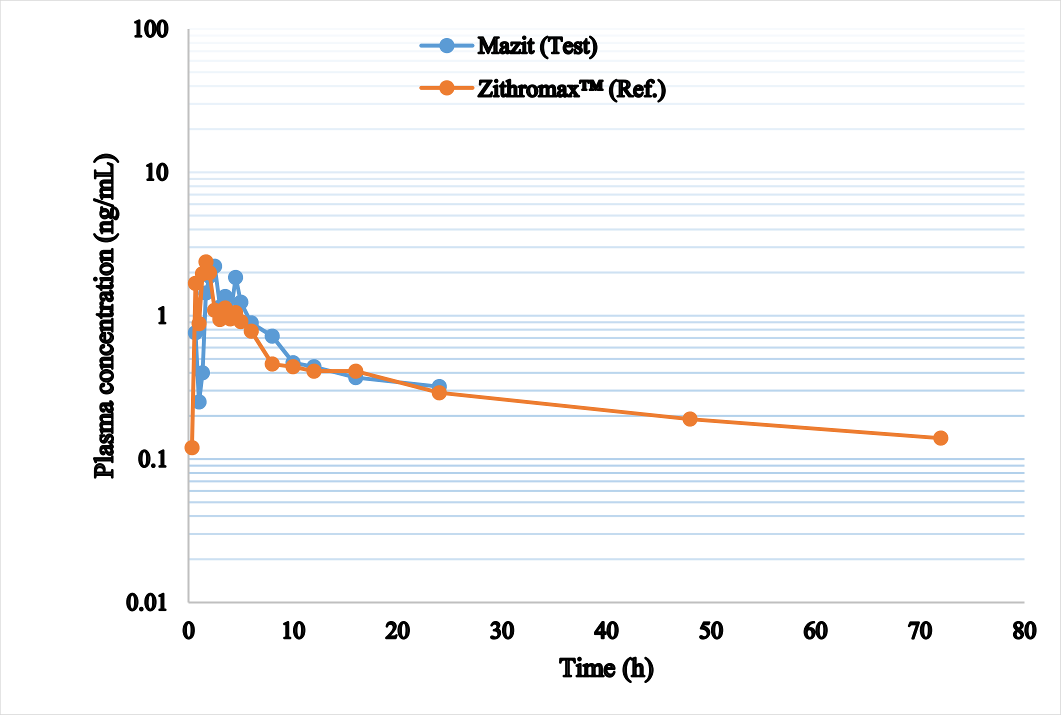

Figure A6.20: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 10)

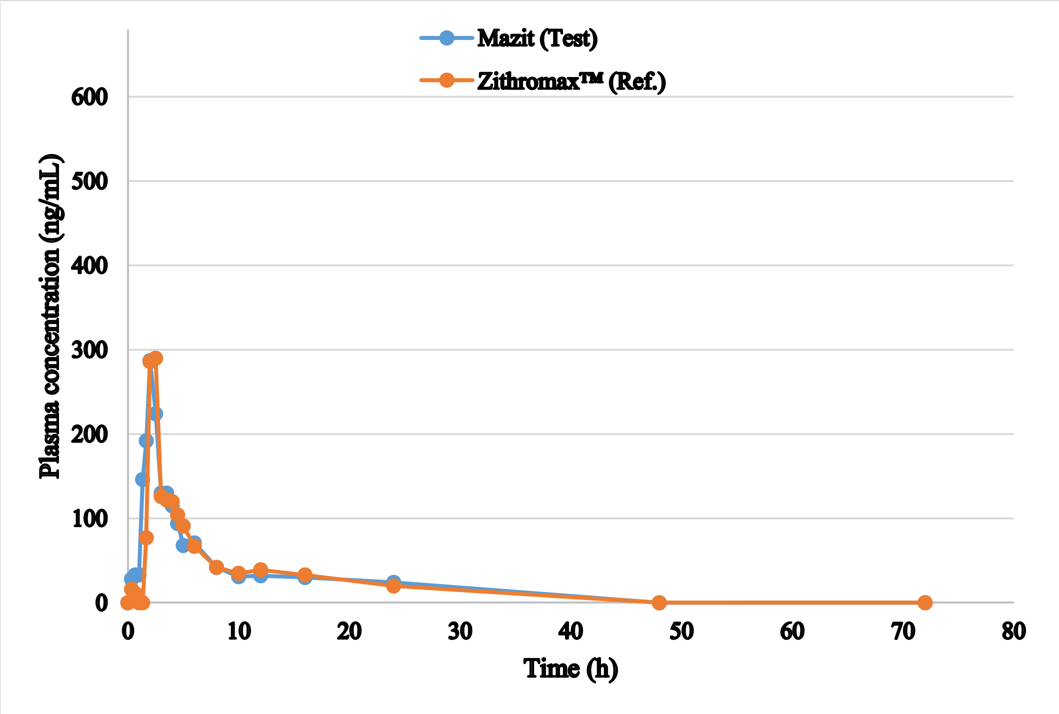

Figure A6.21: Concentration of Mazit compared to Zithromax™ (Subject 11)

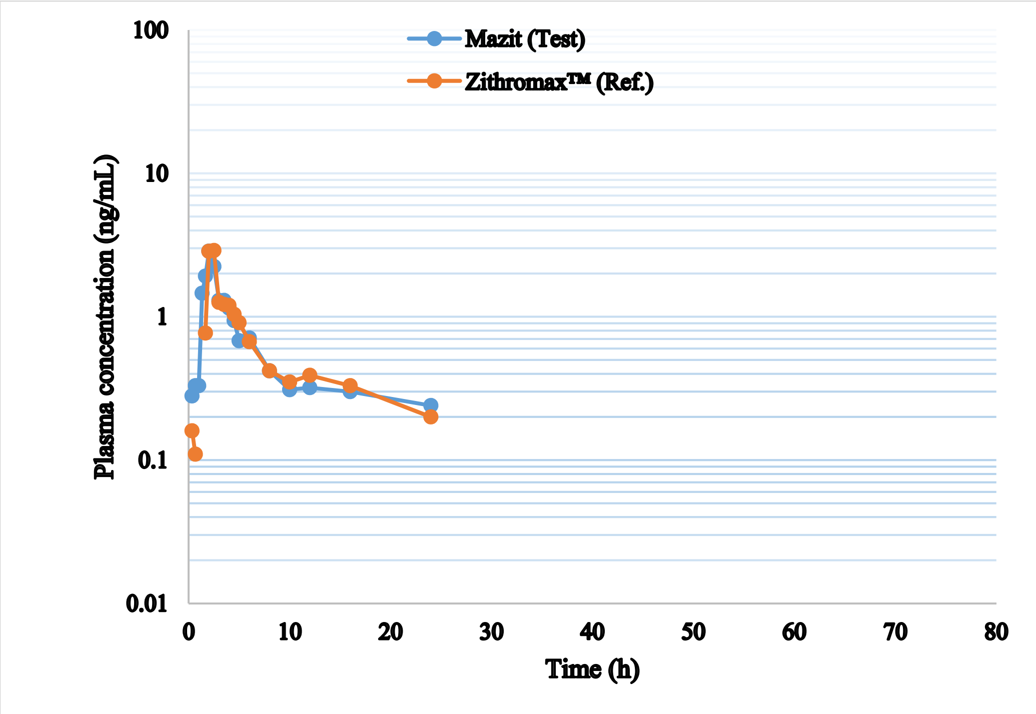

Figure A6.22: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 11)

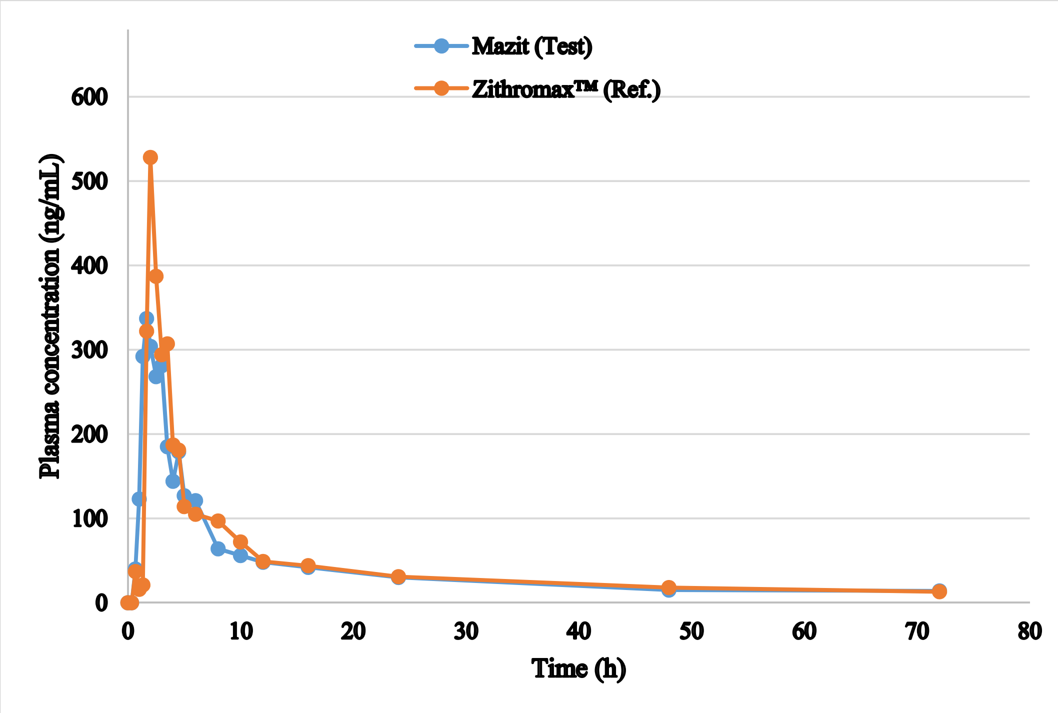

Figure A6.23: Concentration of Mazit compared to Zithromax™ (Subject 12)

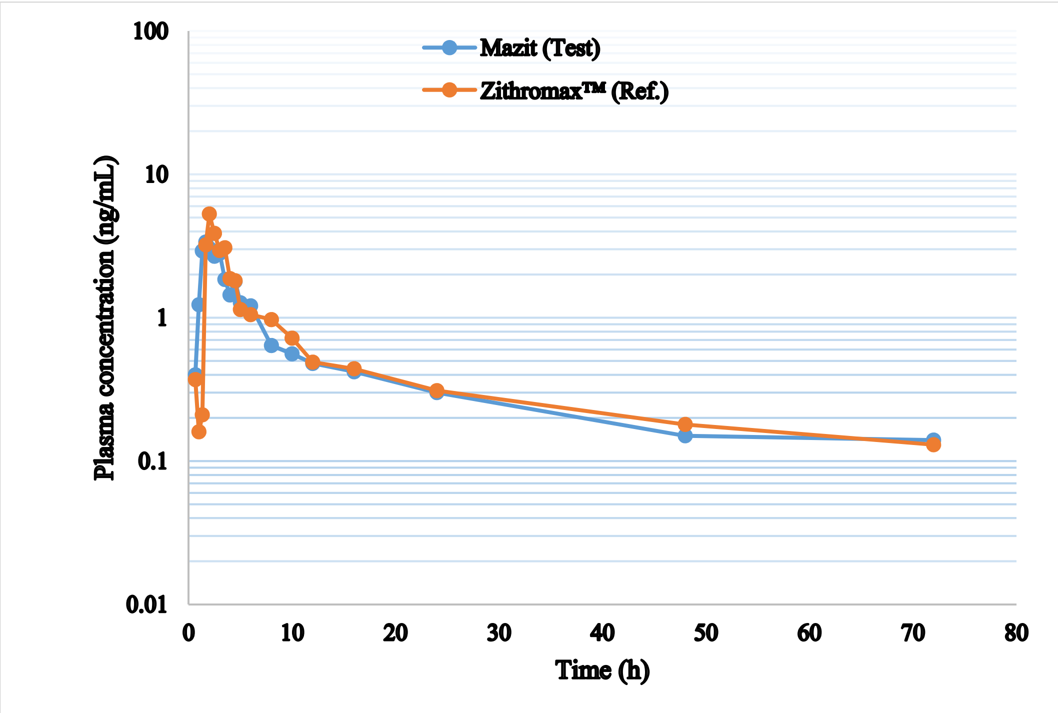

Figure A6.24: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 12)

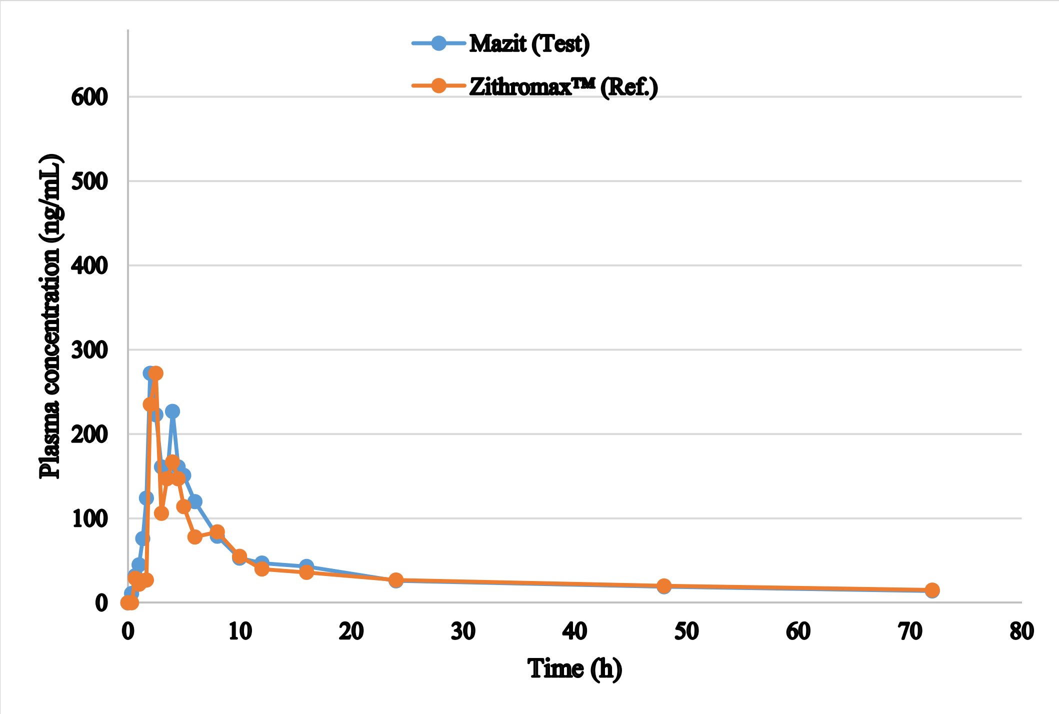

Figure A6.25: Concentration of Mazit compared to Zithromax™ (Subject 13)

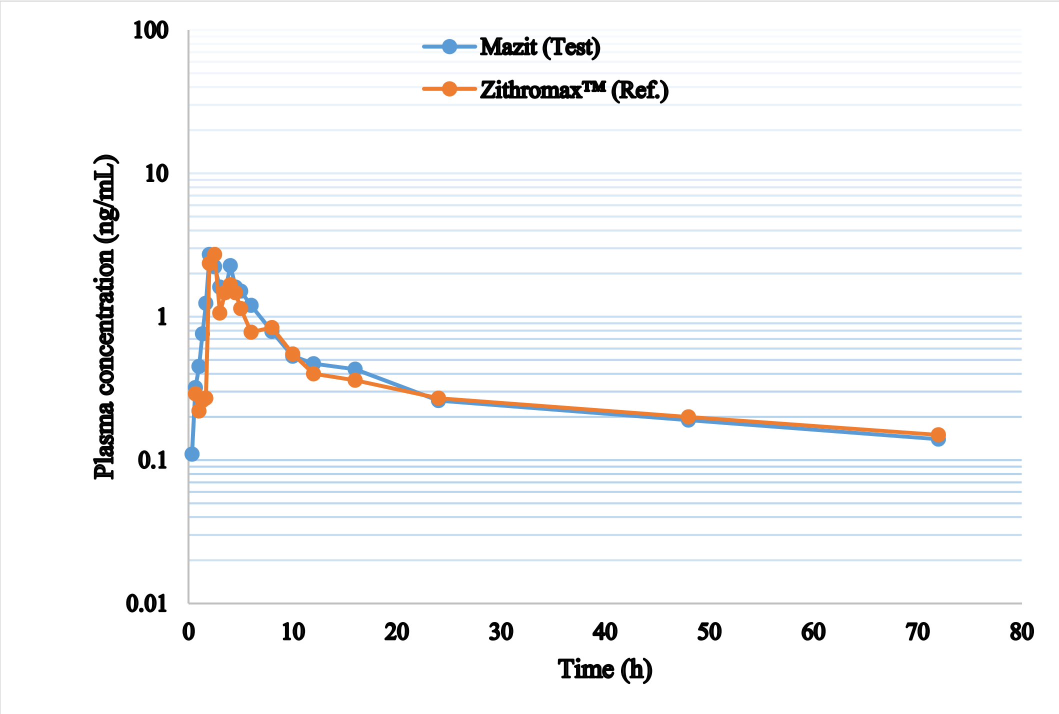

Figure A6.26: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 13)

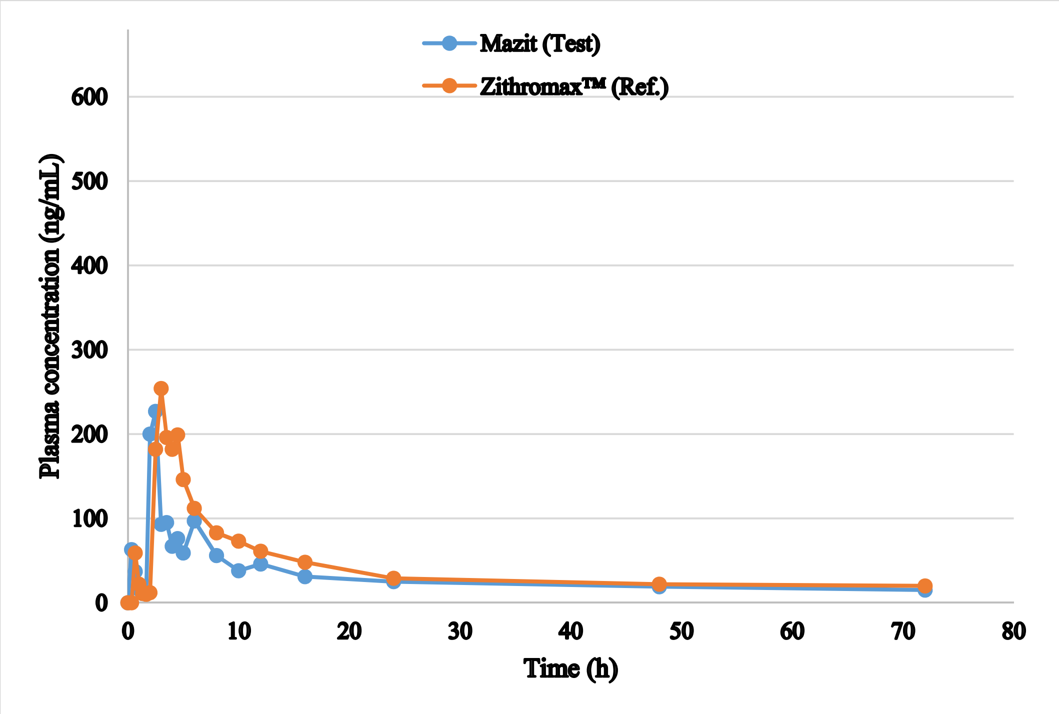

Figure A6.27: Concentration of Mazit compared to Zithromax™ (Subject 14)

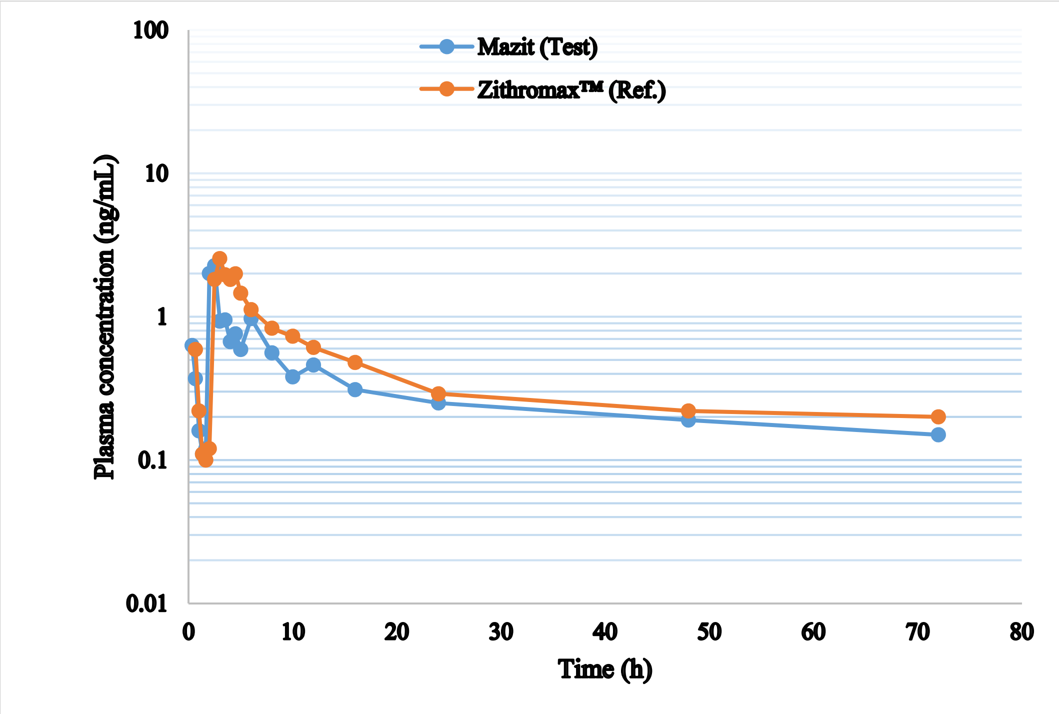

Figure A6.28: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 14)

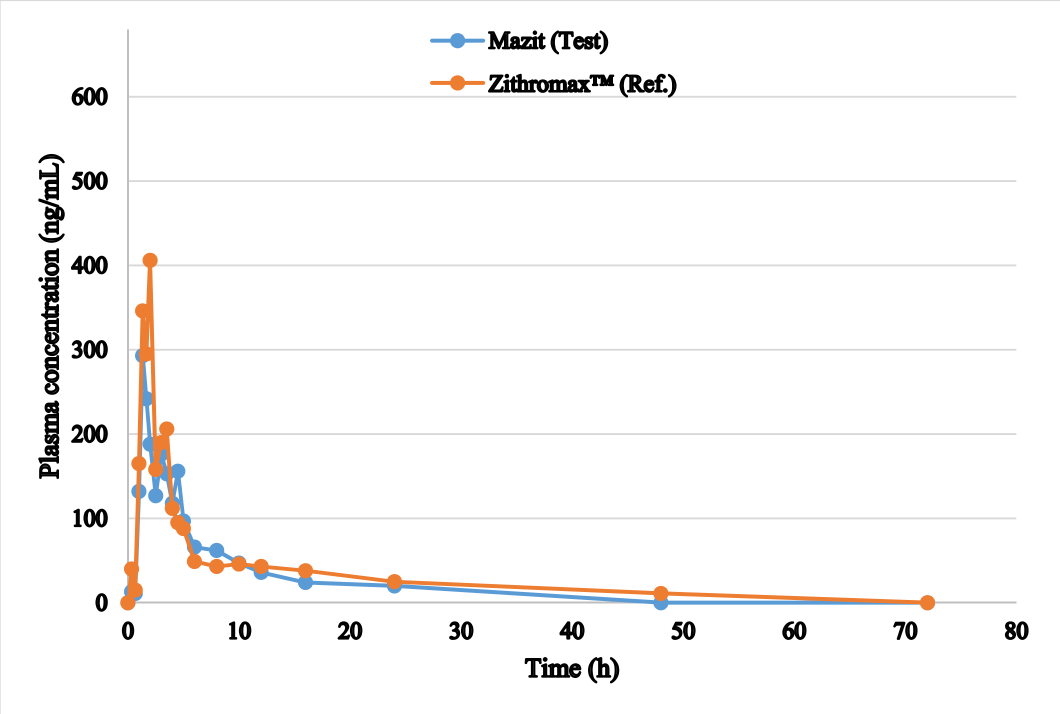

Figure A6.29: Concentration of Mazit compared to Zithromax™ (Subject 15)

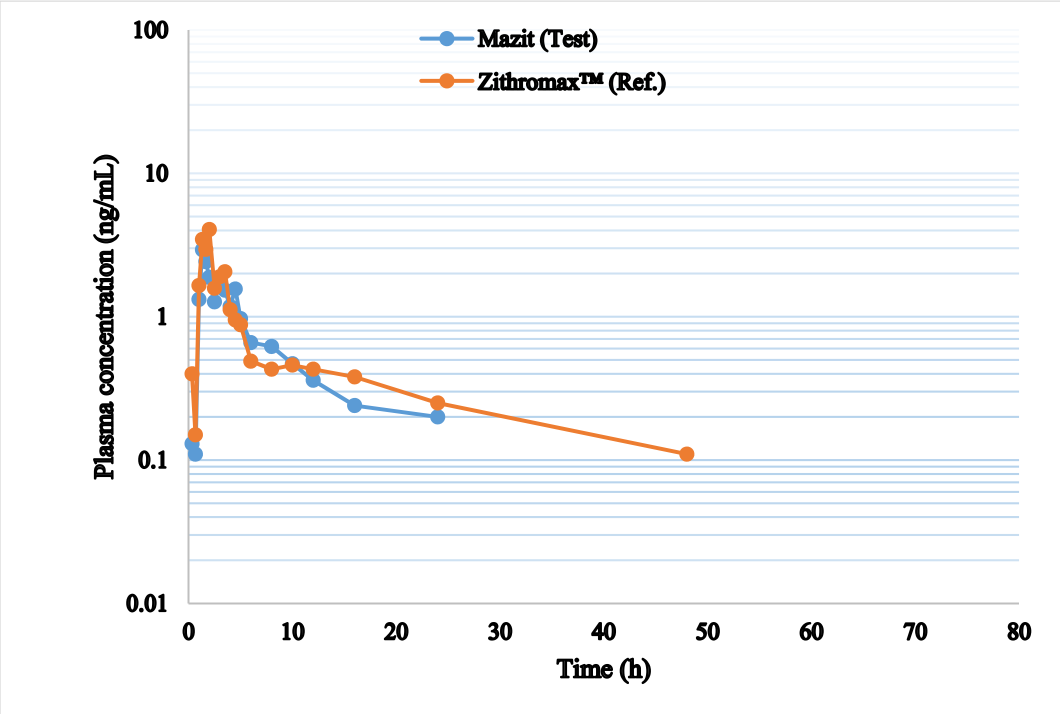

Figure A6.30: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 15)

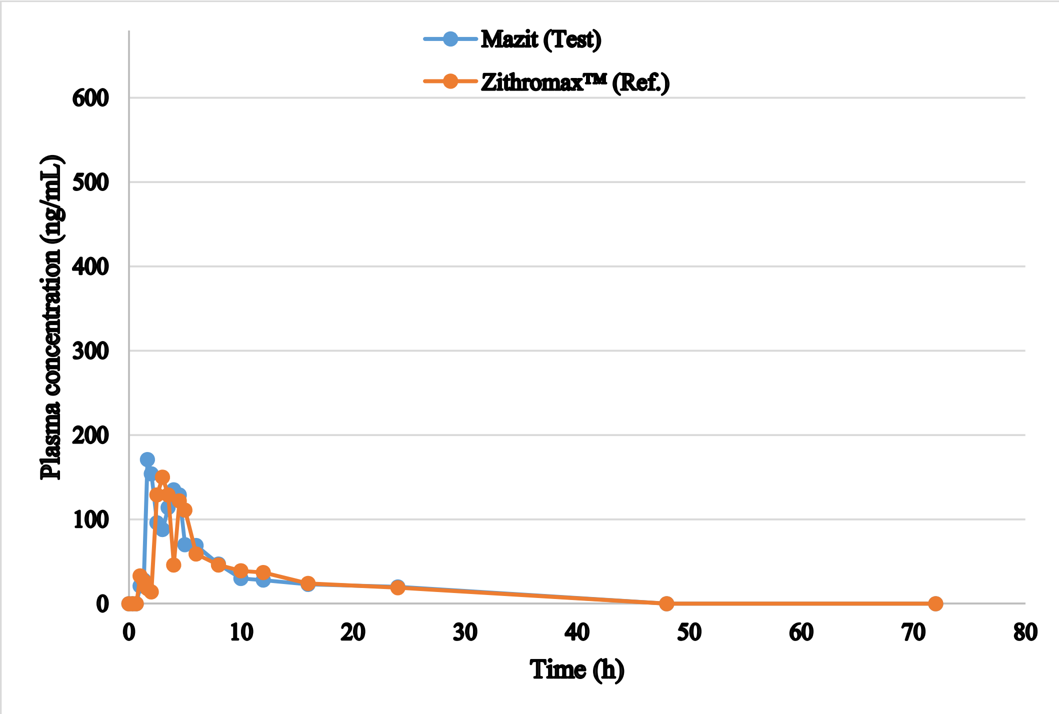

Figure A6.31: Concentration of Mazit compared to Zithromax™ (Subject 16)

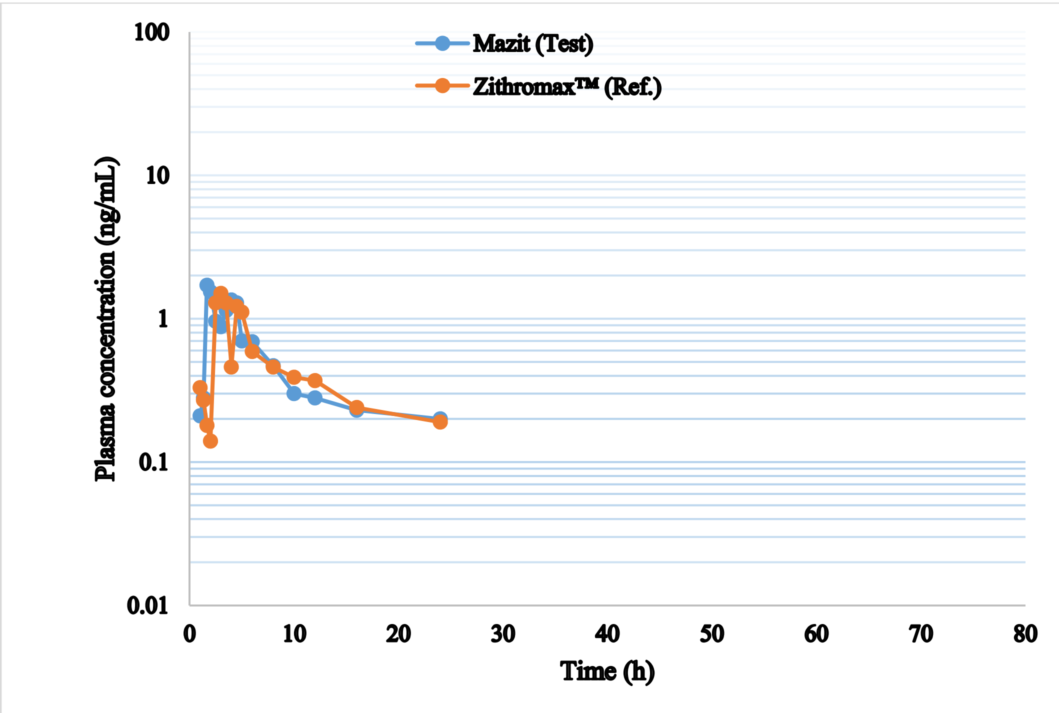

Figure A6.32: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 16)

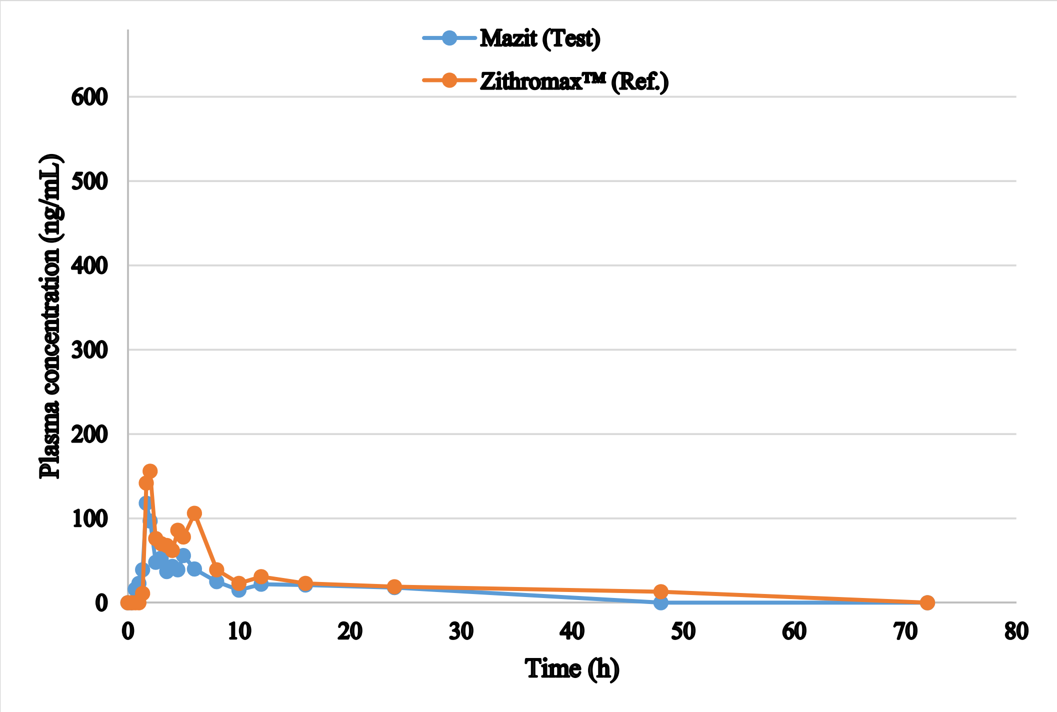

Figure A6.33: Concentration of Mazit compared to Zithromax™ (Subject 17)

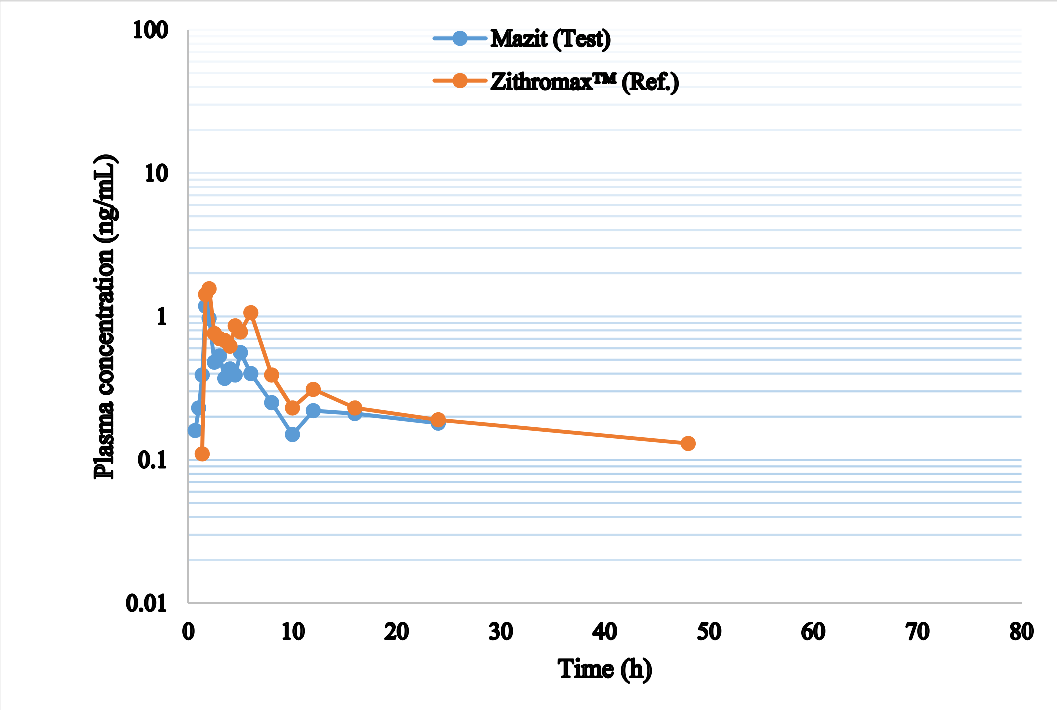

Figure A6.34: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 17)

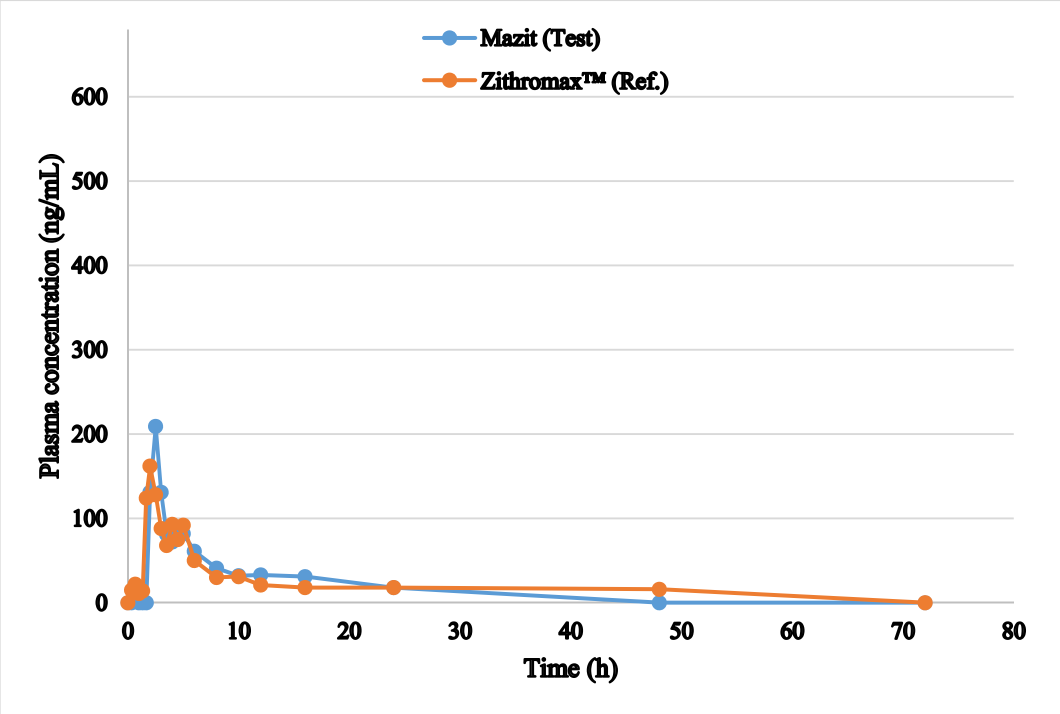

Figure A6.35: Concentration of Mazit compared to Zithromax™ (Subject 18)

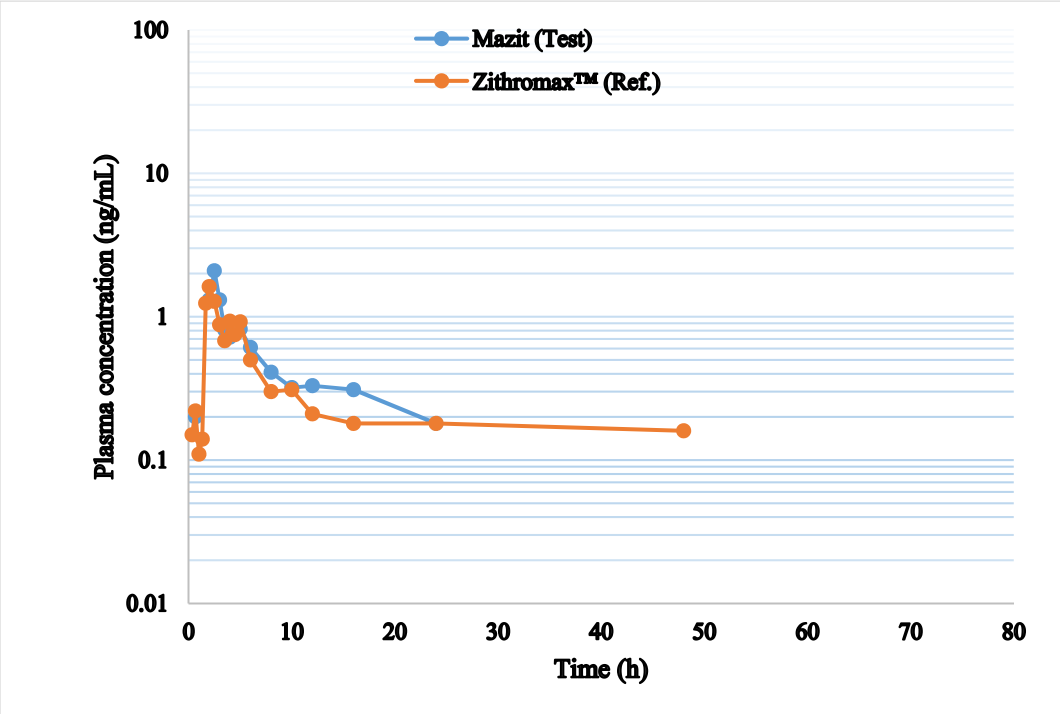

Figure A6.36: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 18)

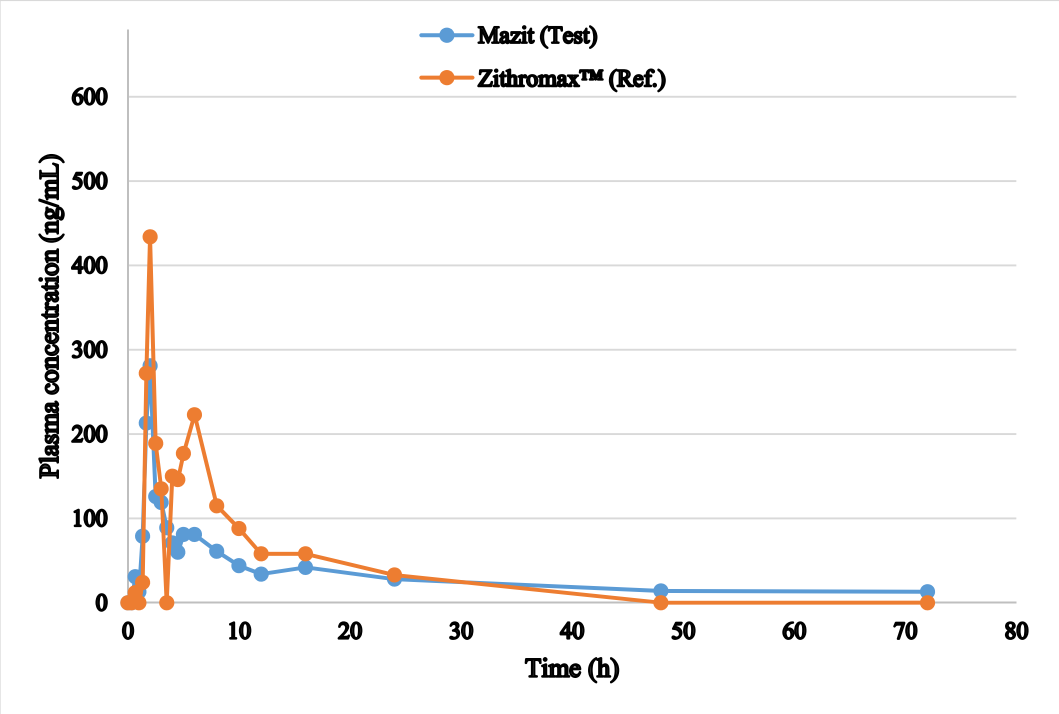

Figure A6.37: Concentration of Mazit compared to Zithromax™ (Subject 19)

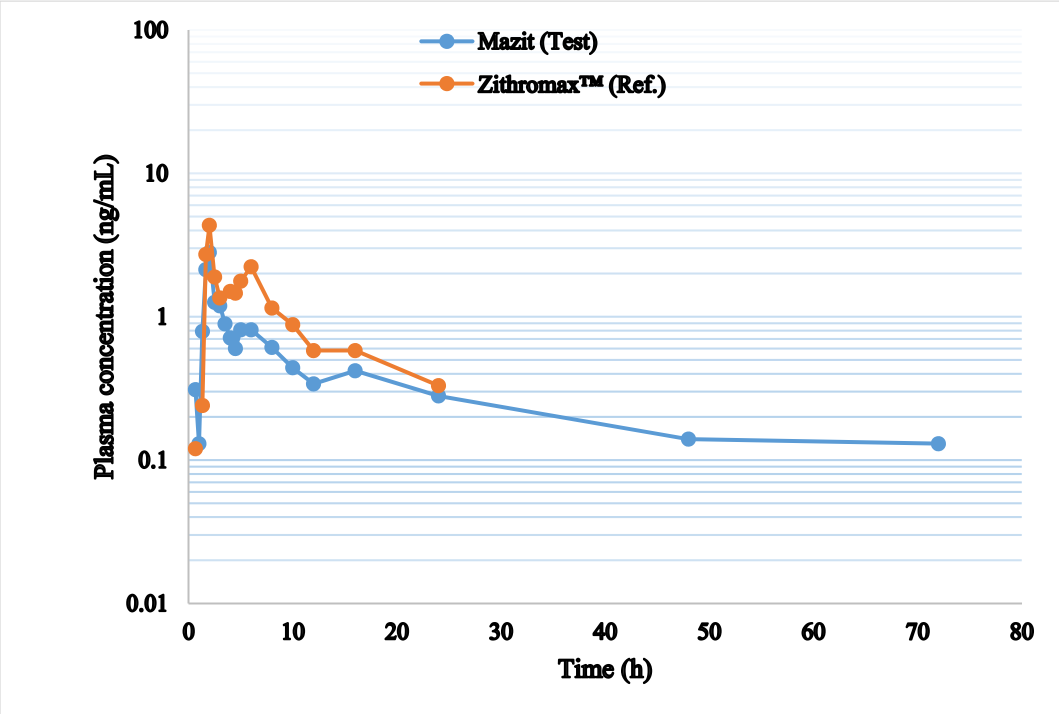

Figure A6.38: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 19)

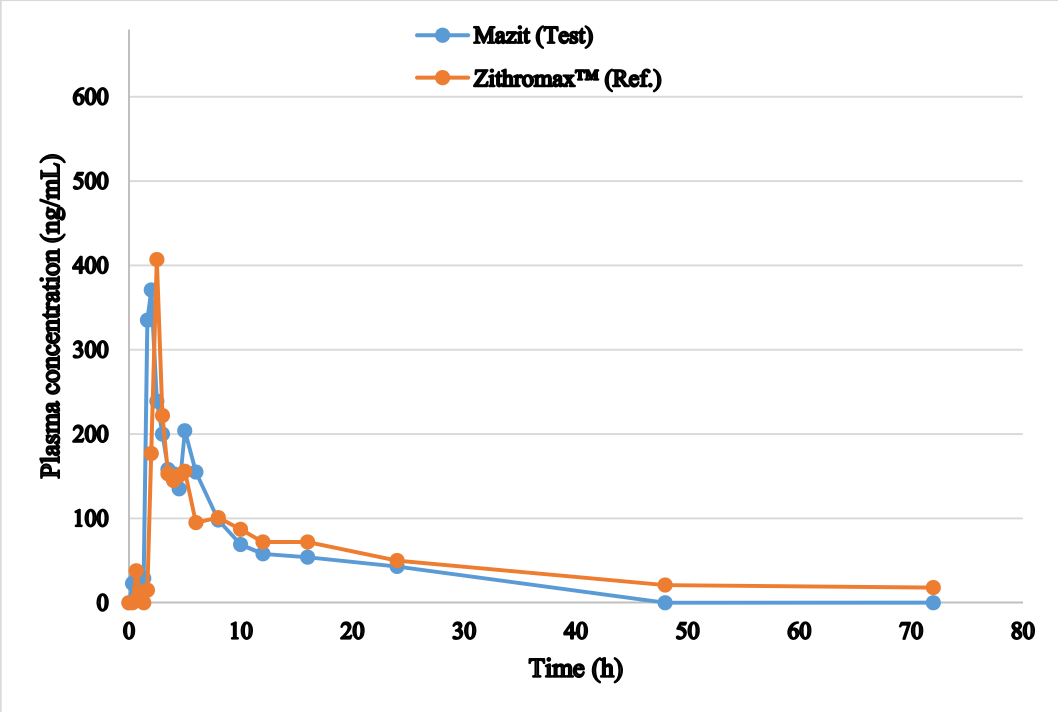

Figure A6.39: Concentration of Mazit compared to Zithromax™ (Subject 20)

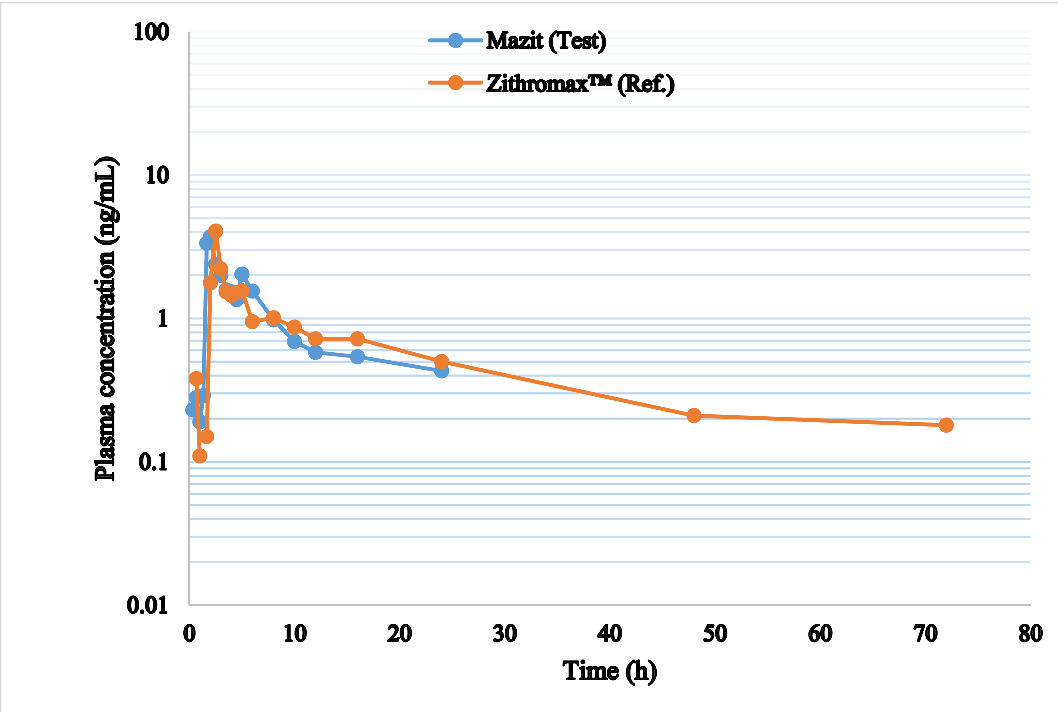

Figure A6.40: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 20)

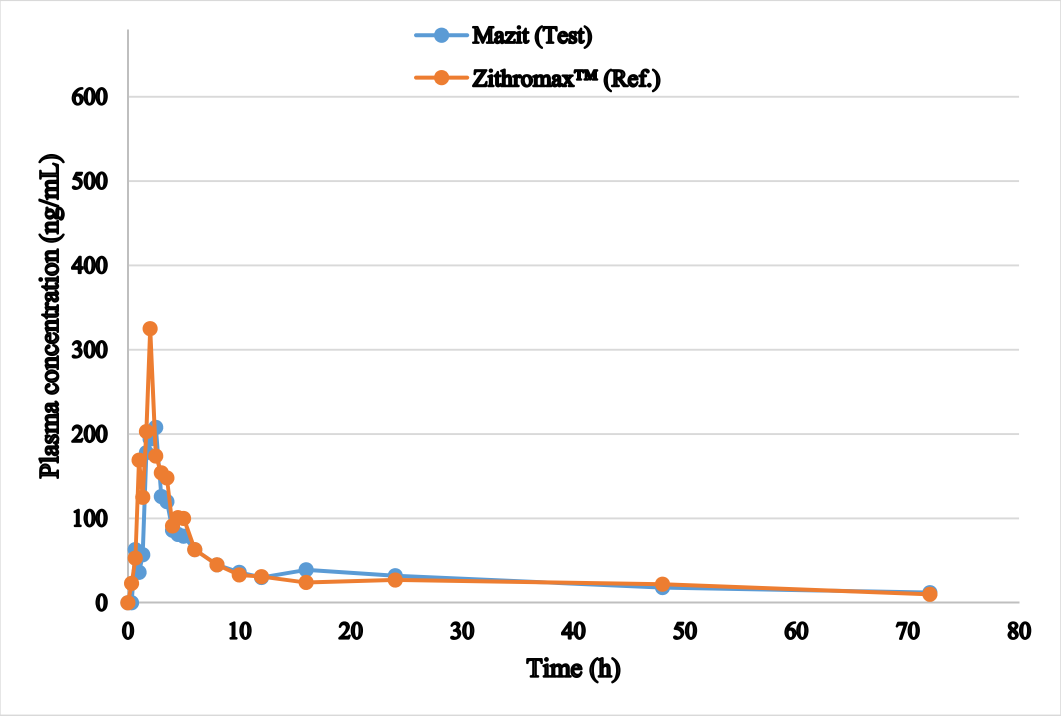

Figure A6.41: Concentration of Mazit compared to Zithromax™ (Subject 21)

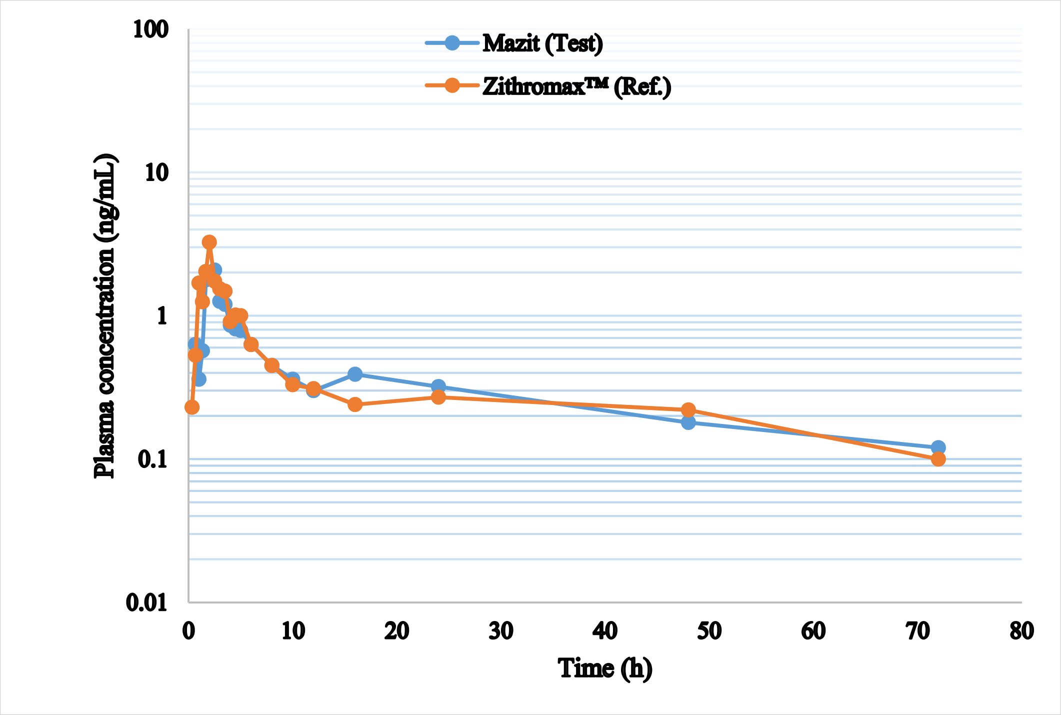

Figure A6.42: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 21)

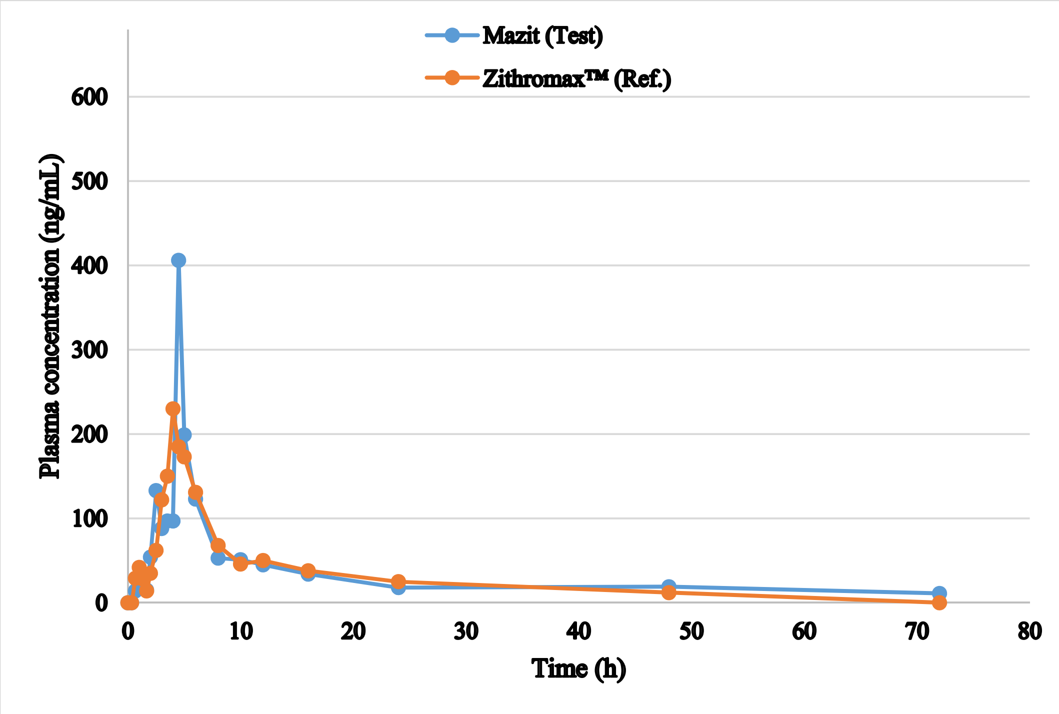

Figure A6.43: Concentration of Mazit compared to Zithromax™ (Subject 22)

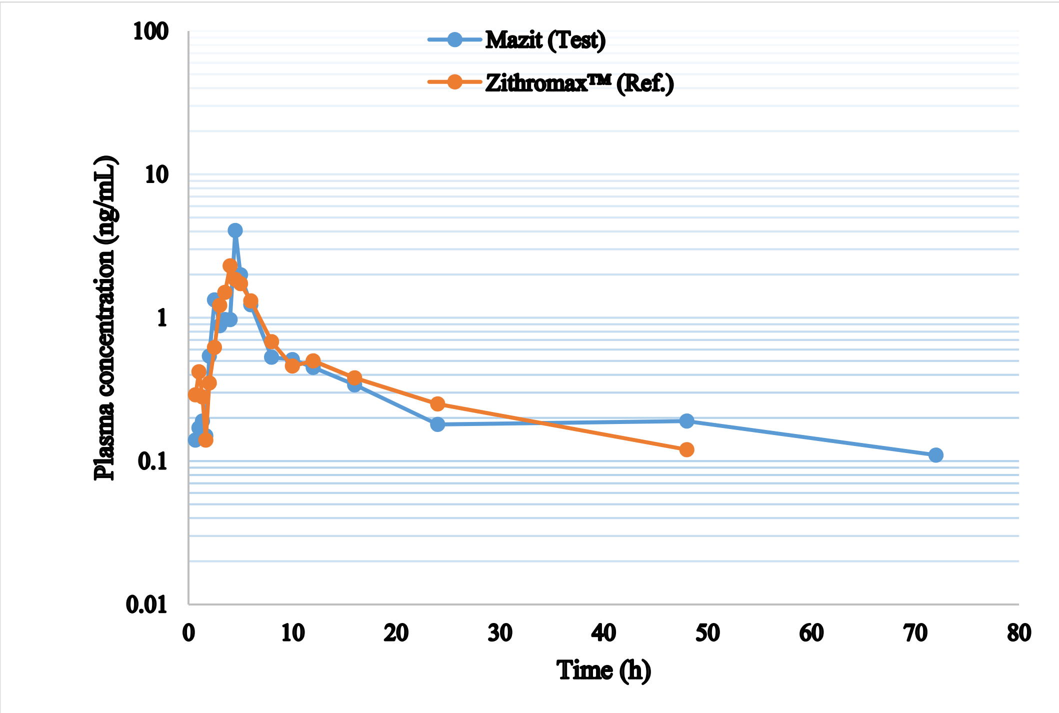

Figure A6.44: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 22)

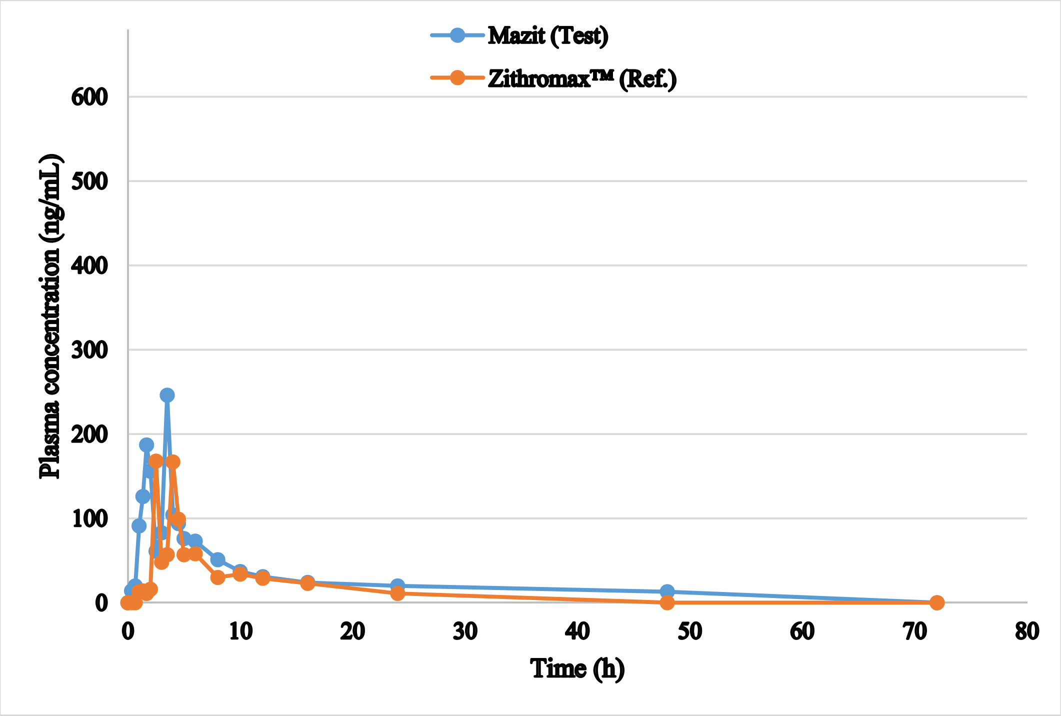

Figure A6.45: Concentration of Mazit compared to Zithromax™ (Subject 23)

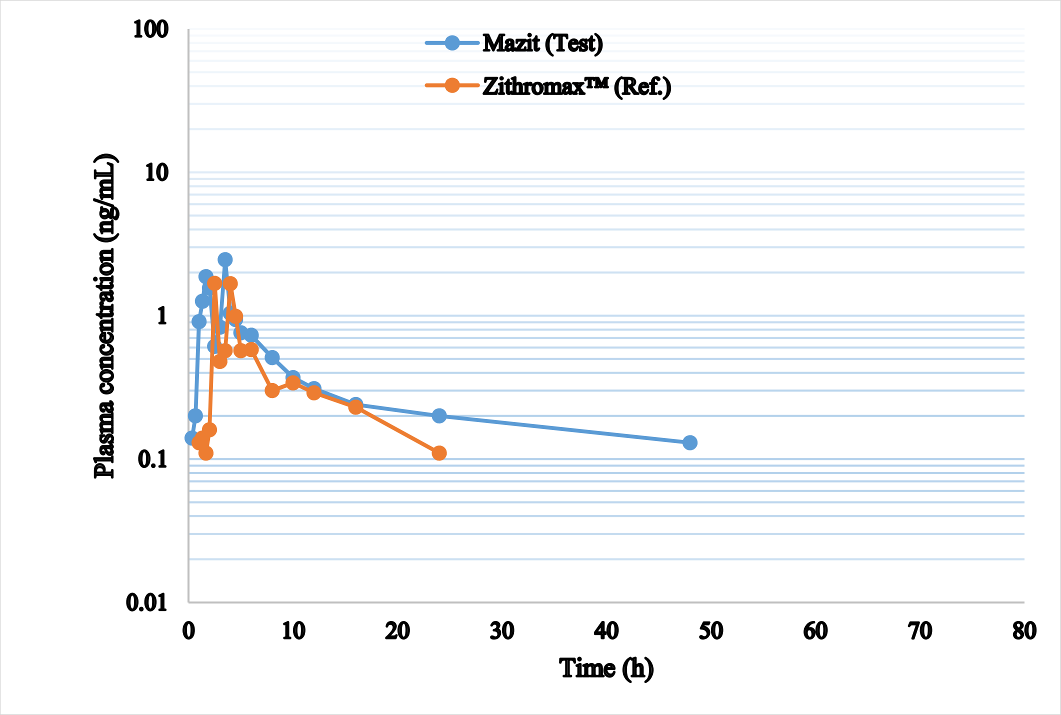

Figure A6.46: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 23)

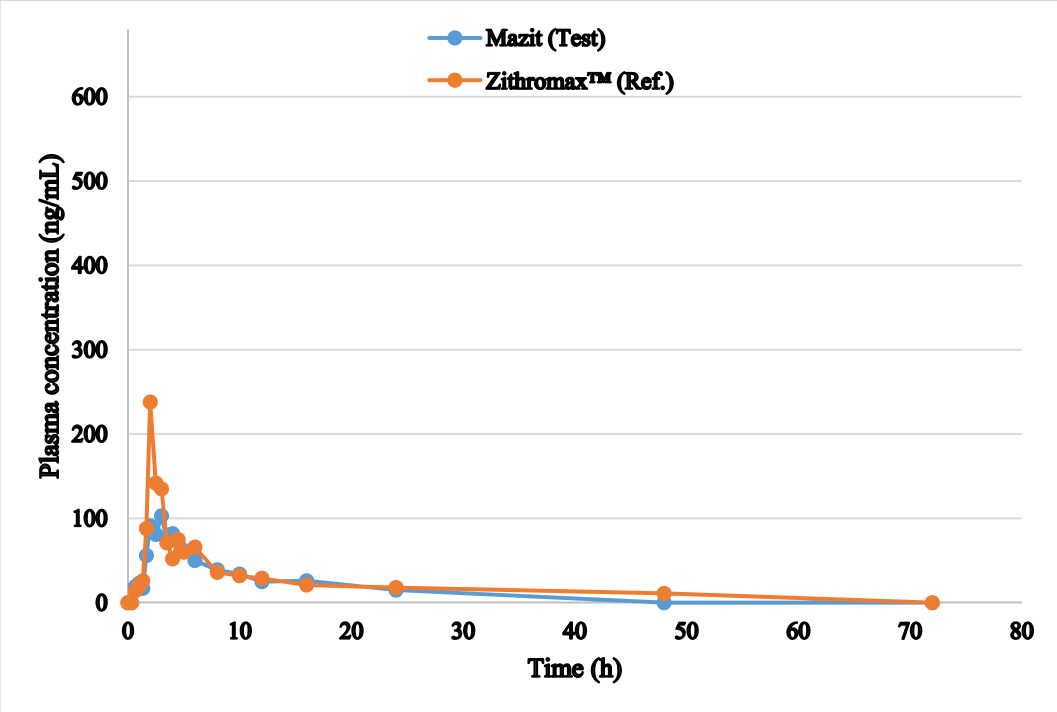

Figure A6.47: Concentration of Mazit compared to Zithromax™ (Subject 24)

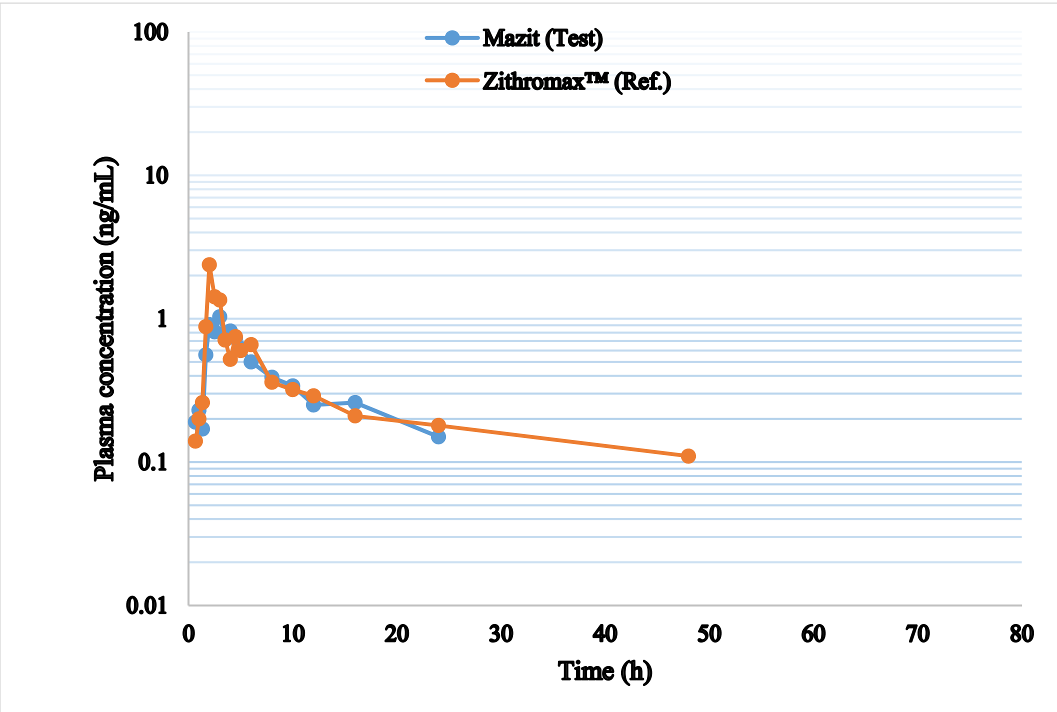

Figure A6.48: Logarithmic concentration of Mazit compared to Zithromax™ (Subject 24)

Appendix 7: Variation from the study protocol for the bioequivalence study between Mazit 250 mg (test product) and Zithromax™ 250 mg (reference product)

Variations From The Clinical Protocol		Subject no.	Impact on the Study Outcome	Definitely none	Most likely none	Possible	Likely	Definite
Clinical lab. Results	HB is slightly below reference range	17,19,27	CS: PK:	☒ ☒	☐	☐	☐	☐
	Crystals are present in urine	02,03,08,19	CS: PK:	☒ ☒	☐	☐	☐	☐
	Mucus is present in urine	01,02,06,08,11, 14,16,18,19,22, 24,25	CS: PK:	☒ ☒	☐	☐	☐	☐
	Leukocytes are present in urine	20	CS: PK:	☒ ☒	☐	☐	☐	☐
	RBC Count is slightly above reference range	04	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sperms are present in urine	19	CS: PK:	☒ ☒	☐	☐	☐	☐
	Neutrophils are slightly below reference range	05	CS: PK:	☒ ☒	☐	☐	☐	☐
	Creatinine is slightly below reference range	15	CS: PK:	☒ ☒	☐	☐	☐	☐
	Specific gravity is slightly above reference range in urine	12,14,22,25	CS: PK:	☒ ☒	☐	☐	☐	☐
	RBC is slightly above reference range in urine	10,17	CS: PK:	☒ ☒	☐	☐	☐	☐
	WBC count is slightly above reference range	12	CS: PK:	☒ ☒	☐	☐	☐	☐
	ketone is slightly above reference range in urine	16	CS: PK:	☒ ☒	☐	☐	☐	☐
	Epithelial cells are present in urine	08	CS: PK:	☒ ☒	☐	☐	☐	☐
	RBC (HB) is present in urine	10	CS: PK:	☒ ☒	☐	☐	☐	☐
	WBC count is slightly below reference range	05	CS: PK:	☒ ☒	☐	☐	☐	☐
Follow up lab. results after period II	ALP is slightly above reference range	09,10,21	CS: PK:	☒ ☒	☐	☐	☐	☐
	ALT is slightly above reference range	03,13	CS: PK:	☒ ☒	☐	☐	☐	☐
	AST is slightly above reference range	15	CS: PK:	☒ ☒	☐	☐	☐	☐
	T.BIL is slightly above reference range	17	CS: PK:	☒ ☒	☐	☐	☐	☐

CS: Clinical safety study outcome.

PK: Pharmacokinetic study outcome.

Variations From The Clinical Protocol		Subject no.		Impact on the Study Outcome				
				Definitely none	Most likely none	Possible	Likely	Definite
Sampling Time Variation (Period I)	Sample no. 19 was withdrawn delayed by 01 hour and 06 minutes	15	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 19 was withdrawn earlier by 01 hour and 04 minutes	23	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 19 was withdrawn earlier by 01 hour and 16 minutes	25	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 19 was withdrawn earlier by 01 hour and 22 minutes	26	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 19 was withdrawn earlier by 01 hour and 16 minutes	27	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 01 minutes	23	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 14 minutes	28	CS: PK:	☒ ☒	☐	☐	☐	☐
Sampling Time Variation (Period II)	Sample no. 19 was withdrawn delayed by 01 hour and 08 minutes	21	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 19 was withdrawn earlier by 01 hour and 03 minutes	27	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 12 minutes	11	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 02 minutes	12	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 08 minutes	13	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 04 minutes	14	CS: PK:	☒ ☒	☐	☐	☐	☐
	Sample no. 20 was withdrawn earlier by 01 hour and 06 minutes	27	CS: PK:	☒ ☒	☐	☐	☐	☐

CS: Clinical safety study outcome.
PK: Pharmacokinetic study outcome.

Appendix 8: The informed consent and other forms used in the bioequivalence study between Mazit 250 mg (test product) and Zithromax™ (reference product)

ANNEX II EXHIBIT # 1
"INFORMED CONSENT FORM" (ENGLISH TRANSLATION)
To be Used In conjunction with SOP: CLP-007 Revision (M)

For IPRC's Use Only	Subject's Initials:	Subject's Number:

The staff of the International Pharmaceutical Research Center (IPRC) has informed me that they wish to perform a research study to compare the plasma drug levels of a new formulation under:

☑ Fasting conditions ☐ Non-fasting conditions

Drug Product under investigation:	**(Mazit Capsules 250 mg)**
Manufacturing Company	**(Neopharma, UAE)**
Reference Drug Product:	**(ZITHROMAX™)**
Manufacturing Company	**PFIZER ITALIA S.r.l., Italy for PFIZER INC., USA.**

I was also informed that I am one of approximately 24 subjects who will participate in this study.

1. Drug Product Information

The active ingredient in the products is	262.05 mg of Azithromycin dihydrate equivalent to 250 mg of azithromycin
It is prescribed	Acute bacterial exacerbations of chronic obstructive pulmonary disease, Acute bacterial sinusitis, Community-acquired pneumonia, Pharyngitis/tonsillitis, Uncomplicated skin and skin structure infections, Urethritis and cervicitis, Genital ulcer disease
It belongs to the class of medications	Macrolides Antibiotic

2. Signatures

I have read the attached pages and I agree to participate in this study . As well, I have received a copy of the ICF (in English language)

Name		*Signature*	*Date*
Participant			
Person who obtained the consent			 ***Time:***
First witness			
ID Number			
Second witness			
ID Number			
The Physician			
Principal Investigator			

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3. Adverse Drug Reactions
The most Common adverse events;

Gastrointestinal tract: diarrhoea, loose stools, abdominal complaints pain, spasm, flatulence, nausea and vomiting , In patients with severe and persistent diarrhoea, the possibility of life-theratening pseudomembraneous colitis should be borne in mind.

Miscellaneous: superinfection with non-susceptible organisms, including fungi.

Rare adverse events:

Hepatobiliary system: A reversible increase in liver enzymes and in serum bilirubin .

Blood and blood corpuscles: neutropenia .

Hypersensitivity reactions: reactions of skin and mucosa such as reddening with or without pruritus, reversible local swellings of skin, mucosa or joints angioedema and acute allergic general reactions (anaphylaxis).

4. Study Procedures

The duration of this study is approximately 25 days and consists of two periods separated by at least a 21-day washout period and the duration of the study might be lengthen if the wash out period lengthen

During Screening:

I will listen to the consenting procedure from the medical team in charge of the study and will give all the necessary information regarding my medical history. I will undergo physical examination, vital signs measurement , ECG. I shall willingly give a blood sample of 13.5 ml to perform blood tests and a urine sample for laboratory examination.

Admission:

Drugs of abuse as well as alcohol screening tests will be performed on admission to both periods and a standardized dinner will be given at least 10 hours before dosing.

During Study Conduction:

I will enter the study facility at least 12 hours before dosing each period and remain there for 24 hours after dosing and I will return back to donate the rest of samples. I understand that while I am in the facility, I will eat and drink only what is provided in the time allotted. Standardized meals will be provided about 4 hours (lunch) and 12 hours (dinner) and a standardized snack will be given 4 hours after lunch. I will be forbidden from drinking water before and after one hour of dosing and then I will be allowed to drink water as I desire. I will remain seated for the first four hours after dosing in each period. Vital signs will be measured before dosing and at the following times after dosing: 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 6^{th}, 8^{th},

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"INFORMED CONSENT FORM" (ENGLISH TRANSLATION)

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11^{th} and 24^{th} hour and at any time if deemed necessary.

During each period, I will be given a 500 mg azithromycin in the form of capsules (two capsules each one containing 262.05 mg of Azithromycin dihydrate equivalent to 250 mg of azithromycin). I shall swallow the drug without chewing with 240 ml of water as a single dose. Treatment assignment (under investigation or reference) in any period will be according to a random scheme. I shall willingly give 22 blood samples in each period (each sample will be 8 ml).

The time schedule for blood sampling (after giving the pre dosing sample and drug dosing):

0.33, 0.66, 1.00, 1.33, 1.66, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10,00, 12.00, 16.00, 24.00, 48.00 and 72.00 hours.

After each blood sampling the cannula will be injected with 0.1 ml of heparin solution its concentration 50 IU/ml (which equals 5 IU) to prevent blood coagulation.

After Study Completion:

I will undergo physical examination, vital signs measurement and ECG. I shall willingly give a blood sample of 9 ml to perform liver function tests .

The total quantity of blood drawn will not exceed 374.5 ml. This volume does not include samples for clinical laboratory repeats, nor samples for ensuring subjects safety based on the judgment of the principal investigator. The total volume should not exceed 420 ml.

I was informed that all the blood samples taken will not be used for any other purposes except those mentioned in the protocol.

I might need to donate urine or blood (not exceeding 10 ml) for repeating any test if needed.

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5. Study's Objectives

The study will evaluate the rate and extent of absorption of the study drug in healthy subjects and is not intended for treatment of a medical condition. As such, this is not an alternative treatment or procedure that is advantageous to the medical condition of the study participants.

6. Benefits

This study has no benefit to my health. If the test formulation was approved for marketing, society may benefit from having this product as a pharmaceutical medication alternate.

7. Qualifications

I have been informed that if my medical history, physical examination and diagnostic laboratory studies have made me eligible for participating in this study, I certify that:

1. I am between 18 to 45 of age.

2. I have informed the staff of IPRC that I didn't participate in any bioequivalence or clinical study within the last two months.

3. I have not donated blood or its derivatives in the past 3 months.

4. I do not have a history of asthma, peptic or gastric ulcer, sinusitis, pharyngitis, renal disorder, hepatic disorder, cardiovascular disorder, neurological disease, haematological disorders or diabetes, psychiatric, dermatologic or immunological disorders.

5. I don't need any medical treatment. I am in a good health that doesn't need medical care.

6. I am not an employee of IPRC and if this was the case my participation or discontinuance will not affect my job performance evaluation.

7. I am not and have never in the past had a known allergy to the drug under study (azithromycin) or any of its other ingredients or any related drug (erythromycin, any macrolide or ketolide antibiotic).

8. I am neither an alcoholic nor am I addicted to any drug.

9. I have fully informed the staff about my medical history and I am aware that hiding any information of this type may be hazardous to my health during the study.

I have also been informed that:

1. I shall not consume any prescription and non-prescription drugs for at least two weeks before starting the study and till the last sample of the study.

2. I shall not consume vitamins (for nutritional purposes) for at least two days before starting the study and

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till the last sample of the study.

3. I will not consume any beverages or food containing methyl-xanthines e.g. caffeine (coffee, tea, cola, energy drinks, chocolate etc.) 24 hours prior to the study drug administration of either Study Period until the end of confinement.

4. I will not consume any beverages or food containing alcohol 48 hours prior to study drug administration until donating the last sample in each respective period.

5. I will not consume any grapefruit containing beverages or food for at least 7 days prior to drug dosing till the last sample of the study .

6. I will not be allowed to engage in strenuous exercise at least one day prior to dosing till the last sample of each respective period of the study.

7. I should not donate blood during this study and for at least 4 weeks after study completion.

8. I may be asked to return for follow-up tests to assure my safety and continued good health.

9. I understand well that I may be discontinued from the study for disruptive behaviour or for violations to the protocol, such as failure to report on time, non compliance to study time schedule, refusal of baggage search as a preventive procedure before or during study conduction, use of drugs, alcohol or behaviour that does not permit the study to be properly performed.

10. I might be withdrawn during any of the study periods without my consent for the benefit off my health and safety as judged by the study physician.

11. The staff at IPRC may want to withdraw blood samples and collect urine samples (the amount of which will be determined by the study physician), if required, for the purpose of establishing my adherence to the above mentioned requirements. I agree to have additional blood and urine samples collected from me, for the above stated reasons and also, if necessary, to ensure my continued good health.

12. Pregnant females or lactating females are excluded from the study (in case there were female participants).

13. I will be excluded if I am taking one or more of the following medication, nelfinavir, warfarin, atorvastatin, carbamazepine, cetirizine, didanosine, efavirenz, fluconazole, indinavir, midazolam, rifabutin, sildenafil, theophylline (intravenous and oral), triazolam, trimethoprim/sulfamethoxazole or zidovudine, digoxin, ergotamine or dihydroergotamine ,terfenadine, cyclosporine, hexobarbital and phenytoin, antacid containing aluminum and magnesium hydroxide, cimetidine.

14. I will be excluded if I have been diagnosed with liver disease, kidney disease, certain heart problems (abnormalities ECG, slow heartbeat, heart failure), family history of certain heart problems, pneumonia, colitis, fungal infection.

15. I will be excluded if I have history of difficulties in swallowing or any gastrointestinal disease which could affect the drug absorption.

EXHIBIT # 1
"INFORMED CONSENT FORM" (ENGLISH TRANSLATION)
To be Used In conjunction with SOP: CLP-007 Revision (M)

8. Voluntary Participation and the Right to Withdraw

I understand that my participation in this study is voluntary and my refusal to participate in this study will not jeopardize my consideration for participation in future studies. I can withdraw from the study at any time and, if so, I will not be penalized <u>however</u>, I shall be compensated as described in section 9.

9. Compensation

During period I of this study, I shall be paid a total of 25% of the compensation for this study, which equals 47.5 JD, During period II of this study, I shall be paid a total of 25% of the compensation for this study, which equals 47.5 JD and 50% by the end of the study (after donating the last study sample) which equals 95 JD. Taking into consideration that the total compensation is 190 JD that include 10 JD transportation fees for each blood sample donated in each visit after leaving the clinical site. I have no rights to ask for any excess compensation.

In case of my withdrawal from the study before the first study drug administration and on my request I shall not have any compensation

In case of termination or suspension of the study by the International Pharmaceutical Research Center or the sponsor I will compensated as follows:

In case of termination or suspension of the study before first study drug administration, I shall be paid a total of 10 JD

In case of termination or suspension of the study before the second study drug administration, I shall be paid a total of 50 % of the compensation for this study which equals 95 JD

In case of termination or suspension of the study in the final period (following final drug administration), I shall be paid in proportion to the period of my participation in the study depending on the number of the donated samples

In case of termination of my study participation for medical reasons and according to study physician decision:

In case of termination of my participation during period I, I shall be paid a total of 10 JD. And if the termination of my participation was done during period II, I shall be paid a total of 47.5 JD

EXHIBIT # 1
"INFORMED CONSENT FORM" (ENGLISH TRANSLATION)
To be Used in conjunction with SOP: CLP-007 Revision (M)

10. Information related to the rights of the subjects

If I have any questions about my related rights as a subject, I can call Dr. Darwish Badran (Chairman of the Institutional Review Board members (Ethical committee responsible for the subjects rights)) who is responsible for subjects rights on the telephone no.: 0795641188

11. Information related to study related injuries

In case of an emergency within the study periods or after I have completed the study, I will first contact the Center where the study was carried out. If I am unable to reach the Center, I will go to the nearest hospital and inform the medical staff there of my participation in a bioequivalence study. In addition, in the case of a study related injury, I will call Dr. Usama Harb (The Principal Investigator and the responsible physician) at: 0776337225

12. Medical Treatment for Study-Related Injury

The International Pharmaceutical Research Center (IPRC) will undertake the full responsibility to complete all the necessary procedures required to procure an insurance contract for the subjects from Euro Arab Insurance Company before conducting the study. Thus, I am fully eligible for treatment of any medical problems that may occur as a result of my participation in this study (the study physician shall determine the extent of the relationship between the injury and the test drug). In the event of a study-related injury, the clinical staff of IPRC will provide immediate medical treatment, free of charge and provide subsequent referrals to the appropriate health care facilities. IPRC will be responsible for the financial coverage of all the medical expenses of injuries suffered as a result of participating in this study.

13. Confidentiality

All records related to my identity and study participation are confidential to the extent required by law and no - one will have access to these records except, the IPRC staff, the sponsor of the study, the Institutional Review Board, Insurance Company, Ministry of Health in Jordan, or any regulatory authority to which this study is submitted to. I have read and understood this consent form which is written in a language that I understand. I have also heard the entire consent form read aloud to me by the IPRC staff. The adverse events that may result from taking this drug have been also explained to me. The staff has answered any questions I have about the study. I agree to participate in this study. And I hold the right to retain a copy of it

IPRC

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

SCREENING RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		SUBJECT NO.	

SUBJECT IDENTIFICATION

Name **First Name** **Middle Name** **Third Name**

Address City Street Tel. No.

Occupation

Date Of Birth				**Age**		❑ Checked from subjects' ID ❑ The Subject was asked

ID Type: ❑ National ID ❑ Passport ______ ❑ Others, *specify* | **Subject ID No.**

Gender ❑ Male ❑ Female

Race ❑ Caucasian ❑ Black ❑ Red ❑ Oriental ❑ Others, *Specify*

Height		Cm	**Weight**		Kg	**Elbow Breadth**		Cm

Body Frame ☑ NA ❑ Small ❑ Medium ❑ Large | **15% of Ideal Weight** ❑ Yes ❑ No

BMI ☑ NA | BMI accepted limits (18-30) ❑ Yes ❑ No

Participation in previous study or donation ❑ No ❑ Yes, *Specify for the last year*

❑ Study Participation *Date* ______ *Volume* ______

❑ Donation *Date* ______ *Volume* ______

❑ Checked from IPRC volunteer database | ❑ Checked from external volunteer database

Performed By: ______ **Date:** ______

MEDICAL DEMOGRAPHICS

Vital Signs After at least 3 minutes sitting

Blood Pressure	mm Hg		/
Pulse	b.p.m.		
Respiration Rate	Per min.		
Temperature	^{0}C		

Performed By: **Date:**

Comments:

Exhibit 2

To be used in conjunction with SOP# DMU-010 (Revision B)

IPRC

CASE REPORT FORM

MEDICAL EVALUATION RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		**SUBJECT NO.**	

MEDICAL DEMOGRAPHICS

Medical History	No	Yes	Notes
1. Abnormal Family history	☐	☐	
2. Allergy (including *drug allergy*)	☐	☐	
3. Abnormal Cardiovascular Functions	☐	☐	
4. Abnormal Respiratory Functions	☐	☐	
5. Abnormal Renal Functions	☐	☐	
6. Abnormal Hepatic Functions	☐	☐	
7. Abnormal Gastrointestinal Functions	☐	☐	
8. Special diet in the last 30 days	☐	☐	
9. Surgery (*If Yes specify Operation and Date)*	☐	☐	
10. Psychiatric disease	☐	☐	
11. Epilepsy or other seizures	☐	☐	
12. Favism (G-6-PD Deficiency)	☐	☐	
13. Bleeding/coagulation disorders or severe anaemia	☐	☐	
14. Acute infection within the last week	☐	☐	
15. Thyroid gland abnormalities	☐	☐	
16. Skin abnormalities	☐	☐	
17. Eye/Ear/Nose/Throat abnormalities	☐	☐	
18. Diabetes	☐	☐	
19. Neurological abnormalities	☐	☐	
20. Musculoskeletal disorders	☐	☐	
21. Immunological diseases	☐	☐	
22. Planned hospitalisation (*within the next 3 months*)	☐	☐	
23. Tobacco use, *if yes specify quantity*	☐	☐	
24. Alcohol consumption, *if yes specify quantity*	☐	☐	☐ Ex-consumer
25. Prescription/OTC Drug Misuse/Abuse, *if yes specify*	☐	☐	
26. Caffeine use, *if yes specify*,	☐	☐	☐ Ex-consumer
	1-☐ coke		______
	2-☐ tea		______
	3-☐ coffee		______
27. Others, *specify*	☐	☐	

Performed By: ______________________ **Date:** ____________

Physician Signature: ______________________ **Date:** ____________

IPRC

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

MEDICAL EVALUATION RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		**SUBJECT NO.**	

PHYSICAL EXAMINATION AND RELATED TESTS

PHYSICAL EXAMINATION	Yes	No	Notes
1. No Abnormalities Detected in Appearance	☐	☐	
2. No Abnormalities Detected in Skin	☐	☐	
3. No Abnormalities Detected in Eyes	☐	☐	
4. No Abnormalities Detected in Ears	☐	☐	
5. No Abnormalities Detected in Nose	☐	☐	
6. No Abnormalities Detected in Throat	☐	☐	
7. No Abnormalities Detected in Mouth	☐	☐	
8. No Abnormalities Detected in Neck	☐	☐	
9. No Abnormalities Detected in Breast	☐	☐	
10. No Abnormalities Detected in Lungs	☐	☐	
11. No Abnormalities Detected in Heart	☐	☐	
12. No Abnormalities Detected in Abdomen	☐	☐	
13. No Abnormalities Detected in Kidneys	☐	☐	
14. No Abnormalities Detected in Spine	☐	☐	
15. No Abnormalities Detected in Lymph nodes	☐	☐	
16. No Abnormalities Detected in Extremities	☐	☐	
17. No Abnormalities Detected in Neurological and Reflex	☐	☐	
18. No Abnormalities Detected in Genitalia	☐	☐	
19. No Abnormalities Detected in Rectum	☐	☐	
20. No Abnormalities Detected in Mental Status	☐	☐	
21. No Abnormalities Detected in ECG Results	☐	☐	
22. No Abnormalities Detected in vital signs measurements	☐	☐	
23. Others, Please specify	☐	☐	

Comments

Performed By: ____________________ **Date:** ____________

IPRC

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

MEDICAL EVALUATION RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		**SUBJECT NO.**	

SELECTION CRITERIA

A-Inclusion Criteria	Yes	No	Notes
1. Age 18 to 45 years, inclusive.	☐	☐	
2. Body weight within 15% of ideal weights for height and weight.	☐	☐	
3. Medical demographics are within the normal range, performed not longer than two weeks before the initiation of the clinical study	☐	☐	
4. Results of laboratory tests are within the normal range. (Laboratory tests are performed not longer than two weeks before the initiation of the clinical study)	☐	☐	
5. Subject does not have a known allergy to the drugs under investigation (azithromycin) or any of its other ingredients or any related drug (erythromycin, any macrolide or ketolide antibiotic).	☐	☐	

B-Exclusion Criteria	No	Yes	Notes
1. Medical demographics with deviations from reference ranges	☐	☐	
2. Results of laboratory tests which are outside the reference ranges	☐	☐	
3. Acute infection within one week preceding first study drug administration	☐	☐	
4. History of drug or alcohol abuse.	☐	☐	
5. Subject is a heavy smoker (more than 10 cigarettes per day).	☐	☐	
6. Subject does not agree not to take any prescription or non-prescription drugs within two weeks before first study drug administration until the end of the study.	☐	☐	
7. Subject does not agree not to take any vitamins taken for nutritional purposes within two days before first study drug administration and until the end of the study.	☐	☐	
8. Subject is on a special diet (for example subject is a vegetarian).	☐	☐	
9. Subject consumes large quantities of alcohol or beverages containing methyl-xanthines e.g. caffeine (coffee, tea, cola, energy drinks, chocolate etc).	☐	☐	
10. Subject does not agree not to consume any beverages or food containing alcohol 48 hours prior to study drug administration until donating the last sample in each respective period.	☐	☐	
11. Subject does not agree not to consume any beverages or food containing methyl-xanthines e.g. caffeine (coffee, tea, cola, energy drinks, chocolate etc.) 24 hours prior to the study Drug administration of either Study Period until the end of confinement.	☐	☐	
12. Subject does not agree not to consume any beverages or food containing grapefruit 7 days prior to first study drug administration until the end of the study.	☐	☐	

Performed By: ____________________ **Date:** ____________

IPRC

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

MEDICAL EVALUATION RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		**SUBJECT NO.**	

SELECTION CRITERIA

B-Exclusion Criteria (Continued)	No	Yes	Notes
13. Subject has a history of severe diseases which have direct impact on the study.	☐	☐	
14. Participation in a bioequivalence study or in a clinical study within the last 2 months before first study drug administration	☐	☐	
15. Subject intends to be hospitalized within 3 months after first study drug administration.	☐	☐	
16. Subjects who, through completion of this study, would have donated more than 500 ml of blood in 14 days, or 750 ml of blood in 30 days, 1000 ml in 90 days, 1250 ml in 120 days, 1500 ml in 180 days, 2000 ml in 270 days, 2500 ml of blood in 1 year.	☐	☐	
17. The subject is a pregnant female (positive urine or blood pregnancy test) or a lactating female in case there were female participants.	☐	☐	
18. Subject has a history or presence of significant asthma, peptic or gastric ulcer, sinusitis, pharyngitis, renal disorder, hepatic disorder, cardiovascular disorder, neurological disease, haematological disorders or diabetes, psychiatric, dermatologic or immunological disorders.	☐	☐	
19. Subject who have been engaged in strenuous exercise at least one day prior to dosing till the last sample of each respective period.	☐	☐	
20. Subjects who have been diagnosed with liver disease, kidney disease, certain heart problems (abnormalities ECG, slow heartbeat, heart failure), family history of certain heart problems, pneumonia, colitis, fungal infection.	☐	☐	
21. Subject is taking one or more of the following medication, nelfinavir, warfarin, atorvastatin, carbamazepine, cetirizine, didanosine, efavirenz, fluconazole, indinavir, midazolam, rifabutin, sildenafil, theophylline (intravenous and oral), triazolam, trimethoprim/sulfamethoxazole or zidovudine, digoxin, ergotamine or dihydroergotamine ,terfenadine, cyclosporine, hexobarbital and phenytoin, antacid containing aluminum and magnesium hydroxide, cimetidine.	☐	☐	
22. Subject has history of difficulties in swallowing or any gastrointestinal disease which could affect the drug absorption.	☐	☐	

Performed By: ____________________ **Date:** ____________

COMMENTS

____

____

____

Performed By: ____________________ **Date:** ____________

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

MEDICAL EVALUATION RECORD

IPRC STUDY CODE			
SUBJECT INITIALS		SUBJECT NO.	

Additional Tests to be performed on screening	*Result*		*Comments*
	Acceptable	*Not Acceptable*	
	❐	❐	
	❐	❐	
	❐	❐	
	❐	❐	
	❐	❐	
	❐	❐	

Recorded By: ____________________ **Date:** ________________

MEDICAL APPROVAL FOR SUBJECT PARTICIPATION

Based on the data contained in the preceding pages I judge this volunteer eligible to participate in this study

❐ Subject can be included ❐ Subject cannot be included

Reason For Exclusion ____

____

Authenticated By: ______________________________ **Date:** ______________

IPRC

Exhibit 3

To be used in conjunction with SOP# DMU-010 (Revision B)

CASE REPORT FORM

FOLLOW UP RECORD AND STUDY CLOSEOUT

IPRC STUDY CODE			
SUBJECT INITIALS		**SUBJECT NO.**	

VITAL SIGNS After at least 3 minutes sitting

Blood Pressure mm Hg ☐ / ☐

Pulse b.p.m. ☐

Respiration Rate Per min. ☐ ***Temperature*** ☐ °C

Performed By ____________________ **Date** ____________

PHYSICAL EXAMINATION	**Yes**	**No**	**Notes**
1. No Abnormalities Detected in Appearance	☐	☐	
2. No Abnormalities Detected in Skin	☐	☐	
3. No Abnormalities Detected in Eyes	☐	☐	
4. No Abnormalities Detected in Ears	☐	☐	
5. No Abnormalities Detected in Nose	☐	☐	
6. No Abnormalities Detected in Throat	☐	☐	
7. No Abnormalities Detected in Mouth	☐	☐	
8. No Abnormalities Detected in Neck	☐	☐	
9. No Abnormalities Detected in Breast	☐	☐	
10. No Abnormalities Detected in Lungs	☐	☐	
11. No Abnormalities Detected in Heart	☐	☐	
12. No Abnormalities Detected in Abdomen	☐	☐	
13. No Abnormalities Detected in Kidneys	☐	☐	
14. No Abnormalities Detected in Spine	☐	☐	
15. No Abnormalities Detected in Lymph nodes	☐	☐	
16. No Abnormalities Detected in Extremities	☐	☐	
17. No Abnormalities Detected in Neurological and Reflex	☐	☐	
18. No Abnormalities Detected in Genitalia	☐	☐	
19. No Abnormalities Detected in Rectum	☐	☐	
20. No Abnormalities Detected in Mental Status	☐	☐	
21. No Abnormalities Detected in ECG Results	☐	☐	
22. No Abnormalities Detected in Vital Signs Measurements	☐	☐	

Performed By: ____________________ **Date:** ____________

23. Other tests ☐* ☐ *See attached sheets

The subject was asked for any adverse events that occurred during the study. The subject

○ left the study without any changes of the baseline condition

○ left the study with some changes of the baseline condition (see relevant Adverse Events forms)

○ Withdrew; ***specify the reason***

○ Study was : ☐ Suspended ☐ Terminated

Performed By: ____________________ **Date:** ____________

Appendix 9: Statistical analysis for the bioequivalence study of Mazit 250 mg (test product) and Zithromax™ 250 mg (reference product)

Minitab Project Report

General Linear Model: LogCmax versus Treatment, Sequence, Period, Subject

```
Method

Factor coding  (-1, 0, +1)


Factor Information

Factor              Type    Levels  Values
Treatment           Fixed        2  Mazit, Zithromax
Sequence            Fixed        2  AB, BA
Period              Fixed        2  1, 2
Subject(Sequence)   Random      24  2(AB), 3(AB), 5(AB), 10(AB), 11(AB), 12(AB),
13(AB),
                                    14(AB), 15(AB), 16(AB), 20(AB), 23(AB),
1(BA), 4(BA),
                                    6(BA), 7(BA), 8(BA), 9(BA), 17(BA), 18(BA),
19(BA),
                                    21(BA), 22(BA), 24(BA)


Analysis of Variance

Source               DF   Adj SS    Adj MS  F-Value  P-Value
  Treatment           1  0.00345  0.003450     0.21    0.655
  Sequence            1  0.13548  0.135483     3.33    0.082
  Period              1  0.00218  0.002182     0.13    0.722
  Subject(Sequence)  22  0.89572  0.040714     2.42    0.022
Error                22  0.36957  0.016798
Total                47  1.40640


Model Summary

       S    R-sq  R-sq(adj)  R-sq(pred)
0.129609  73.72%     43.86%       0.00%


Coefficients

Term                  Coef  SE Coef  T-Value  P-Value   VIF
Constant            2.4150   0.0187   129.09    0.000
Treatment
  Mazit            -0.0085   0.0187    -0.45    0.655  1.00
Sequence
  AB                0.0531   0.0187     2.84    0.010  1.00
Period
  1                 0.0067   0.0187     0.36    0.722  1.00
Subject(Sequence)
```

```
  2(AB)            0.1614   0.0877     1.84    0.079     *
  3(AB)            0.0086   0.0877     0.10    0.923     *
  5(AB)            0.1436   0.0877     1.64    0.116     *
  10(AB)          -0.1086   0.0877    -1.24    0.229     *
  11(AB)          -0.0080   0.0877    -0.09    0.928     *
  12(AB)           0.1570   0.0877     1.79    0.087     *
  13(AB)          -0.0336   0.0877    -0.38    0.706     *
  14(AB)          -0.0877   0.0877    -1.00    0.328     *
  15(AB)           0.0695   0.0877     0.79    0.436     *
  16(AB)          -0.2636   0.0877    -3.00    0.007     *
  20(AB)           0.1213   0.0877     1.38    0.181     *
  1(BA)           -0.1575   0.0877    -1.79    0.086     *
  4(BA)           -0.0632   0.0877    -0.72    0.479     *
  6(BA)            0.0522   0.0877     0.59    0.558     *
  7(BA)            0.2156   0.0877     2.46    0.022     *
  8(BA)            0.1403   0.0877     1.60    0.124     *
  9(BA)           -0.0513   0.0877    -0.58    0.565     *
  17(BA)          -0.2294   0.0877    -2.61    0.016     *
  18(BA)          -0.0971   0.0877    -1.11    0.281     *
  19(BA)           0.1812   0.0877     2.07    0.051     *
  21(BA)           0.0531   0.0877     0.60    0.551     *
  22(BA)           0.1232   0.0877     1.40    0.174     *


Regression Equation

LogCmax = 2.4150 - 0.0085 Treatment_Mazit + 0.0085 Treatment_Zithromax
+ 0.0531 Sequence_AB
          - 0.0531 Sequence_BA + 0.0067 Period_1 - 0.0067 Period_2
          + 0.1614 Subject(Sequence)_2(AB) + 0.0086 Subject(Sequence)_3(AB)
          + 0.1436 Subject(Sequence)_5(AB) - 0.1086 Subject(Sequence)_10(AB)
          - 0.0080 Subject(Sequence)_11(AB) + 0.1570 Subject(Sequence)_12(AB)
          - 0.0336 Subject(Sequence)_13(AB) - 0.0877 Subject(Sequence)_14(AB)
          + 0.0695 Subject(Sequence)_15(AB) - 0.2636 Subject(Sequence)_16(AB)
          + 0.1213 Subject(Sequence)_20(AB) - 0.1600 Subject(Sequence)_23(AB)
          - 0.1575 Subject(Sequence)_1(BA) - 0.0632 Subject(Sequence)_4(BA)
          + 0.0522 Subject(Sequence)_6(BA) + 0.2156 Subject(Sequence)_7(BA)
          + 0.1403 Subject(Sequence)_8(BA) - 0.0513 Subject(Sequence)_9(BA)
          - 0.2294 Subject(Sequence)_17(BA) - 0.0971 Subject(Sequence)_18(BA)
          + 0.1812 Subject(Sequence)_19(BA) + 0.0531 Subject(Sequence)_21(BA)
          + 0.1232 Subject(Sequence)_22(BA) - 0.1672 Subject(Sequence)_24(BA)

Equation treats random terms as though they are fixed.


Fits and Diagnostics for Unusual Observations

Obs  LogCmax     Fit    Resid  Std Resid
  2   2.8136  2.6279   0.1857       2.12  R
 26   2.4456  2.6313  -0.1857      -2.12  R

R  Large residual


Expected Mean Squares, using Adjusted SS

                      Expected Mean Square
   Source             for Each Term
1  Treatment          (5) + Q[1]
2  Sequence           (5) + 2.0000 (4) + Q[2]
3  Period             (5) + Q[3]
4  Subject(Sequence)  (5) + 2.0000 (4)
5  Error              (5)
```

Error Terms for Tests, using Adjusted SS

	Source	Error DF	Error MS	Synthesis of Error MS
1	Treatment	22.00	0.0168	(5)
2	Sequence	22.00	0.0407	(4)
3	Period	22.00	0.0168	(5)
4	Subject(Sequence)	22.00	0.0168	(5)

Variance Components, using Adjusted SS

Source	Variance	% of Total	StDev	% of Total
Subject(Sequence)	0.0119580	41.58%	0.109353	64.49%
Error	0.0167985	58.42%	0.129609	76.43%
Total	0.0287565		0.169577	

Comparisons for LogCmax

Dunnett Multiple Comparisons with a Control: Response = LogCmax, Term = Treatment

Grouping Information Using the Dunnett Method and 90% Confidence

Treatment	N	Mean	Grouping
Zithromax (Control)	24	2.42350	A
Mazit	24	2.40654	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 90% CI	T-Value	Adjusted P-Value
Mazit - Zithromax	-0.0170	0.0374	(-0.0812, 0.0473)	-0.45	0.655

Individual confidence level = 90.00%

General Linear Model: LogAUC0-t versus Treatment, Sequence, Period, Subject

Method

Factor coding (-1, 0, +1)

Factor Information

Factor	Type	Levels	Values
Treatment	Fixed	2	Mazit, Zithromax
Sequence	Fixed	2	AB, BA
Period	Fixed	2	1, 2
Subject(Sequence)	Random	24	2(AB), 3(AB), 5(AB), 10(AB), 11(AB), 12(AB), 13(AB), 14(AB), 15(AB), 16(AB), 20(AB), 23(AB), 1(BA), 4(BA), 6(BA), 7(BA), 8(BA), 9(BA), 17(BA), 18(BA), 19(BA), 21(BA), 22(BA), 24(BA)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	1	0.01120	0.011198	1.01	0.326
Sequence	1	0.07908	0.079077	1.44	0.243
Period	1	0.00710	0.007104	0.64	0.433
Subject(Sequence)	22	1.20804	0.054911	4.94	0.000
Error	22	0.24457	0.011117		
Total	47	1.54999			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.105437	84.22%	66.29%	24.89%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.2305	0.0152	212.27	0.000	
Treatment					
Mazit	-0.0153	0.0152	-1.00	0.326	1.00
Sequence					
AB	0.0406	0.0152	2.67	0.014	1.00
Period					
1	0.0122	0.0152	0.80	0.433	1.00
Subject(Sequence)					
2(AB)	-0.0369	0.0714	-0.52	0.610	*
3(AB)	-0.0373	0.0714	-0.52	0.606	*
5(AB)	0.1474	0.0714	2.06	0.051	*
10(AB)	0.0069	0.0714	0.10	0.924	*
11(AB)	-0.1673	0.0714	-2.34	0.029	*
12(AB)	0.2008	0.0714	2.81	0.010	*
13(AB)	0.1358	0.0714	1.90	0.070	*
14(AB)	0.1196	0.0714	1.68	0.108	*
15(AB)	-0.0434	0.0714	-0.61	0.549	*
16(AB)	-0.2660	0.0714	-3.73	0.001	*
20(AB)	0.1616	0.0714	2.26	0.034	*

```
  1(BA)           -0.2694   0.0714    -3.77    0.001     *
  4(BA)           -0.1194   0.0714    -1.67    0.108     *
  6(BA)            0.0220   0.0714     0.31    0.761     *
  7(BA)            0.2461   0.0714     3.45    0.002     *
  8(BA)            0.1339   0.0714     1.88    0.074     *
  9(BA)            0.0756   0.0714     1.06    0.301     *
  17(BA)          -0.2174   0.0714    -3.05    0.006     *
  18(BA)          -0.1366   0.0714    -1.91    0.069     *
  19(BA)           0.1468   0.0714     2.06    0.052     *
  21(BA)           0.1668   0.0714     2.34    0.029     *
  22(BA)           0.1222   0.0714     1.71    0.101     *


Regression Equation

LogAUC0-t = 3.2305 - 0.0153 Treatment_Mazit + 0.0153 Treatment_Zithromax
+ 0.0406 Sequence_AB
            - 0.0406 Sequence_BA + 0.0122 Period_1 - 0.0122 Period_2
            - 0.0369 Subject(Sequence)_2(AB) - 0.0373 Subject(Sequence)_3(AB)
            + 0.1474 Subject(Sequence)_5(AB) + 0.0069 Subject(Sequence)_10(AB)
            - 0.1673 Subject(Sequence)_11(AB) + 0.2008 Subject(Sequence)_12(AB)
            + 0.1358 Subject(Sequence)_13(AB) + 0.1196 Subject(Sequence)_14(AB)
            - 0.0434 Subject(Sequence)_15(AB) - 0.2660 Subject(Sequence)_16(AB)
            + 0.1616 Subject(Sequence)_20(AB) - 0.2211 Subject(Sequence)_23(AB)
            - 0.2694 Subject(Sequence)_1(BA) - 0.1194 Subject(Sequence)_4(BA)
            + 0.0220 Subject(Sequence)_6(BA) + 0.2461 Subject(Sequence)_7(BA)
            + 0.1339 Subject(Sequence)_8(BA) + 0.0756 Subject(Sequence)_9(BA)
            - 0.2174 Subject(Sequence)_17(BA) - 0.1366 Subject(Sequence)_18(BA)
            + 0.1468 Subject(Sequence)_19(BA) + 0.1668 Subject(Sequence)_21(BA)
            + 0.1222 Subject(Sequence)_22(BA) - 0.1705 Subject(Sequence)_24(BA)

Equation treats random terms as though they are fixed.


Fits and Diagnostics for Unusual Observations

Obs  LogAUC0-t     Fit    Resid  Std Resid
  2     3.3783  3.2310   0.1472       2.06  R
 26     3.0900  3.2372  -0.1472      -2.06  R

R  Large residual


Expected Mean Squares, using Adjusted SS

                      Expected Mean Square
   Source             for Each Term
1  Treatment          (5) + Q[1]
2  Sequence           (5) + 2.0000 (4) + Q[2]
3  Period             (5) + Q[3]
4  Subject(Sequence)  (5) + 2.0000 (4)
5  Error              (5)


Error Terms for Tests, using Adjusted SS

                                          Synthesis
   Source             Error DF  Error MS  of Error MS
1  Treatment             22.00    0.0111  (5)
2  Sequence              22.00    0.0549  (4)
3  Period                22.00    0.0111  (5)
4  Subject(Sequence)     22.00    0.0111  (5)
```

Variance Components, using Adjusted SS

Source	Variance	% of Total	StDev	% of Total
Subject(Sequence)	0.0218969	66.33%	0.147976	81.44%
Error	0.0111169	33.67%	0.105437	58.03%
Total	0.0330138		0.181697	

Comparisons for LogAUC0-t

Dunnett Multiple Comparisons with a Control: Response = LogAUC0-t, Term = Treatment

Grouping Information Using the Dunnett Method and 90% Confidence

Treatment	N	Mean	Grouping
Zithromax (Control)	24	3.24575	A
Mazit	24	3.21520	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 90% CI	T-Value	Adjusted P-Value
Mazit - Zithromax	-0.0305	0.0304	(-0.0828, 0.0217)	-1.00	0.326

Individual confidence level = 90.00%

General Linear Model: LogAUC0inf versus Treatment, Sequence, Period, Subject

Method

```
Factor coding  (-1, 0, +1)
Rows unused    2
```

Factor Information

```
Factor             Type    Levels  Values
Treatment          Fixed        2  Mazit, Zithromax
Sequence           Fixed        2  AB, BA
Period             Fixed        2  1, 2
Subject(Sequence)  Random      24  2(AB), 3(AB), 5(AB), 10(AB), 11(AB), 12(AB),
13(AB),
                                   14(AB), 15(AB), 16(AB), 20(AB), 23(AB),
1(BA), 4(BA),
                                   6(BA), 7(BA), 8(BA), 9(BA), 17(BA), 18(BA),
19(BA),
                                   21(BA), 22(BA), 24(BA)
```

Analysis of Variance

```
Source              DF   Adj SS    Adj MS  F-Value  P-Value
  Treatment          1  0.00919  0.009194     0.54    0.470
  Sequence           1  0.06337  0.063370     1.40    0.250  x
  Period             1  0.05592  0.055915     3.30    0.084
  Subject(Sequence) 22  1.02460  0.046573     2.75    0.013
Error               20  0.33853  0.016927
Total               45  1.45701
```

x Not an exact F-test.

Model Summary

```
       S    R-sq  R-sq(adj)  R-sq(pred)
0.130103  76.77%     47.72%           *
```

Coefficients

```
Term                  Coef  SE Coef  T-Value  P-Value   VIF
Constant            3.3653   0.0196   171.58    0.000
Treatment
  Mazit            -0.0145   0.0196    -0.74    0.470  1.04
Sequence
  AB                0.0380   0.0196     1.93    0.067  1.05
Period
  1                 0.0356   0.0196     1.82    0.084  1.05
Subject(Sequence)
  2(AB)            -0.1134   0.0884    -1.28    0.214     *
  3(AB)             -0.098    0.125    -0.79    0.439     *
  5(AB)             0.0943   0.0884     1.07    0.299     *
  10(AB)            0.0703   0.0884     0.80    0.436     *
  11(AB)           -0.1228   0.0884    -1.39    0.180     *
  12(AB)            0.1551   0.0884     1.75    0.095     *
  13(AB)            0.1644   0.0884     1.86    0.078     *
  14(AB)            0.2358   0.0884     2.67    0.015     *
```

```
  15(AB)          -0.1074   0.0884    -1.21    0.239     *
  16(AB)          -0.2288   0.0884    -2.59    0.018     *
  20(AB)           0.1934   0.0884     2.19    0.041     *
  1(BA)            -0.292    0.125    -2.35    0.029     *
  4(BA)           -0.1911   0.0884    -2.16    0.043     *
  6(BA)           -0.0173   0.0884    -0.20    0.847     *
  7(BA)            0.1923   0.0884     2.17    0.042     *
  8(BA)            0.1131   0.0884     1.28    0.216     *
  9(BA)            0.0223   0.0884     0.25    0.803     *
  17(BA)          -0.0512   0.0884    -0.58    0.569     *
  18(BA)           0.0993   0.0884     1.12    0.275     *
  19(BA)           0.1127   0.0884     1.27    0.217     *
  21(BA)           0.1211   0.0884     1.37    0.186     *
  22(BA)           0.0632   0.0884     0.71    0.483     *
```

```
Regression Equation

LogAUC0inf = 3.3653 - 0.0145 Treatment_Mazit + 0.0145 Treatment_Zithromax
             + 0.0380 Sequence_AB - 0.0380 Sequence_BA + 0.0356 Period_1
- 0.0356 Period_2
             - 0.1134 Subject(Sequence)_2(AB) - 0.098 Subject(Sequence)_3(AB)
             + 0.0943 Subject(Sequence)_5(AB) + 0.0703 Subject(Sequence)_10(AB)
             - 0.1228 Subject(Sequence)_11(AB)
+ 0.1551 Subject(Sequence)_12(AB)
             + 0.1644 Subject(Sequence)_13(AB)
+ 0.2358 Subject(Sequence)_14(AB)
             - 0.1074 Subject(Sequence)_15(AB)
- 0.2288 Subject(Sequence)_16(AB)
             + 0.1934 Subject(Sequence)_20(AB)
- 0.2427 Subject(Sequence)_23(AB)
             - 0.292 Subject(Sequence)_1(BA) - 0.1911 Subject(Sequence)_4(BA)
             - 0.0173 Subject(Sequence)_6(BA) + 0.1923 Subject(Sequence)_7(BA)
             + 0.1131 Subject(Sequence)_8(BA) + 0.0223 Subject(Sequence)_9(BA)
             - 0.0512 Subject(Sequence)_17(BA)
+ 0.0993 Subject(Sequence)_18(BA)
             + 0.1127 Subject(Sequence)_19(BA)
+ 0.1211 Subject(Sequence)_21(BA)
             + 0.0632 Subject(Sequence)_22(BA)
- 0.1720 Subject(Sequence)_24(BA)

Equation treats random terms as though they are fixed.


Fits and Diagnostics for Unusual Observations

Obs  LogAUC0inf    Fit   Resid  Std Resid
 18       3.128  3.377  -0.248      -2.83  R
 25       3.085  3.085   0.000          *     X
 27       3.284  3.284   0.000          *     X
 42       3.725  3.477   0.248       2.83  R

R  Large residual
X  Unusual X


Expected Mean Squares, using Adjusted SS

                      Expected Mean Square
   Source             for Each Term
1  Treatment          (5) + Q[1]
2  Sequence           (5) + 1.8333 (4) + Q[2]
3  Period             (5) + Q[3]
```

```
4  Subject(Sequence)  (5) + 1.9091 (4)
5  Error              (5)
```

Error Terms for Tests, using Adjusted SS

	Source	Error DF	Error MS	Synthesis of Error MS
1	Treatment	20.00	0.0169	(5)
2	Sequence	22.66	0.0454	0.9603 (4) + 0.0397 (5)
3	Period	20.00	0.0169	(5)
4	Subject(Sequence)	20.00	0.0169	(5)

Variance Components, using Adjusted SS

Source	Variance	% of Total	StDev	% of Total
Subject(Sequence)	0.0155289	47.85%	0.124615	69.17%
Error	0.0169267	52.15%	0.130103	72.22%
Total	0.0324556		0.180154	

Comparisons for LogAUC0inf

Dunnett Multiple Comparisons with a Control: Response = LogAUC0inf, Term = Treatment

Grouping Information Using the Dunnett Method and 90% Confidence

Treatment	N	Mean	Grouping
Zithromax (Control)	24	3.37973	A
Mazit	22	3.35082	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 90% CI	T-Value	Adjusted P-Value
Mazit - Zithromax	-0.0289	0.0392	(-0.0966, 0.0387)	-0.74	0.470

Individual confidence level = 90.00%

General Linear Model: Logt½ versus Treatment, Sequence, Period, Subject

Method

Factor coding	(-1, 0, +1)
Rows unused	2

Factor Information

Factor	Type	Levels	Values
Treatment	Fixed	2	Mazit, Zithromax
Sequence	Fixed	2	AB, BA
Period	Fixed	2	1, 2
Subject(Sequence)	Random	24	2(AB), 3(AB), 5(AB), 10(AB), 11(AB), 12(AB), 13(AB), 14(AB), 15(AB), 16(AB), 20(AB), 23(AB), 1(BA), 4(BA), 6(BA), 7(BA), 8(BA), 9(BA), 17(BA), 18(BA), 19(BA), 21(BA), 22(BA), 24(BA)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Treatment	1	0.00468	0.004677	0.07	0.787	
Sequence	1	0.00000	0.000000	0.00	1.000	x
Period	1	0.11383	0.113830	1.82	0.193	
Subject(Sequence)	22	2.29016	0.104098	1.66	0.129	
Error	20	1.25300	0.062650			
Total	45	3.66324				

x Not an exact F-test.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.250300	65.80%	23.04%	*

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	1.3916	0.0377	36.88	0.000	
Treatment					
Mazit	-0.0103	0.0377	-0.27	0.787	1.04
Sequence					
AB	0.0000	0.0377	0.00	1.000	1.05
Period					
1	0.0509	0.0377	1.35	0.193	1.05
Subject(Sequence)					
2(AB)	-0.258	0.170	-1.52	0.145	*
3(AB)	-0.232	0.240	-0.97	0.345	*
5(AB)	0.088	0.170	0.52	0.610	*
10(AB)	0.140	0.170	0.82	0.422	*
11(AB)	-0.122	0.170	-0.72	0.481	*
12(AB)	0.134	0.170	0.79	0.440	*
13(AB)	0.345	0.170	2.03	0.056	*
14(AB)	0.486	0.170	2.85	0.010	*
15(AB)	-0.269	0.170	-1.58	0.129	*

```
  16(AB)           -0.179    0.170    -1.05    0.305     *
  20(AB)            0.023    0.170     0.13    0.894     *
  1(BA)            -0.221    0.240    -0.92    0.367     *
  4(BA)            -0.540    0.170    -3.17    0.005     *
  6(BA)             0.024    0.170     0.14    0.887     *
  7(BA)             0.090    0.170     0.53    0.601     *
  8(BA)             0.149    0.170     0.88    0.391     *
  9(BA)             0.044    0.170     0.26    0.798     *
  17(BA)            0.213    0.170     1.25    0.225     *
  18(BA)            0.288    0.170     1.69    0.106     *
  19(BA)           -0.103    0.170    -0.60    0.552     *
  21(BA)            0.136    0.170     0.80    0.434     *
  22(BA)           -0.004    0.170    -0.02    0.982     *


Regression Equation

Logt½ = 1.3916 - 0.0103 Treatment_Mazit + 0.0103 Treatment_Zithromax
+ 0.0000 Sequence_AB
        - 0.0000 Sequence_BA + 0.0509 Period_1 - 0.0509 Period_2
        - 0.258 Subject(Sequence)_2(AB) - 0.232 Subject(Sequence)_3(AB)
        + 0.088 Subject(Sequence)_5(AB) + 0.140 Subject(Sequence)_10(AB)
        - 0.122 Subject(Sequence)_11(AB) + 0.134 Subject(Sequence)_12(AB)
        + 0.345 Subject(Sequence)_13(AB) + 0.486 Subject(Sequence)_14(AB)
        - 0.269 Subject(Sequence)_15(AB) - 0.179 Subject(Sequence)_16(AB)
        + 0.023 Subject(Sequence)_20(AB) - 0.155 Subject(Sequence)_23(AB)
        - 0.221 Subject(Sequence)_1(BA) - 0.540 Subject(Sequence)_4(BA)
        + 0.024 Subject(Sequence)_6(BA) + 0.090 Subject(Sequence)_7(BA)
        + 0.149 Subject(Sequence)_8(BA) + 0.044 Subject(Sequence)_9(BA)
        + 0.213 Subject(Sequence)_17(BA) + 0.288 Subject(Sequence)_18(BA)
        - 0.103 Subject(Sequence)_19(BA) + 0.136 Subject(Sequence)_21(BA)
        - 0.004 Subject(Sequence)_22(BA) - 0.078 Subject(Sequence)_24(BA)

Equation treats random terms as though they are fixed.


Fits and Diagnostics for Unusual Observations

Obs  Logt½    Fit   Resid  Std Resid
 18  1.117  1.618  -0.502      -2.97  R
 25  1.231  1.231  -0.000          *     X
 27  1.119  1.119  -0.000          *     X
 42  2.243  1.741   0.502       2.97  R

R  Large residual
X  Unusual X


Expected Mean Squares, using Adjusted SS

                      Expected Mean Square
   Source             for Each Term
1  Treatment          (5) + Q[1]
2  Sequence           (5) + 1.8333 (4) + Q[2]
3  Period             (5) + Q[3]
4  Subject(Sequence)  (5) + 1.9091 (4)
5  Error              (5)


Error Terms for Tests, using Adjusted SS

   Source             Error DF  Error MS  Synthesis of Error MS
1  Treatment             20.00    0.0626  (5)
```

```
2  Sequence              23.09    0.1025  0.9603 (4) + 0.0397 (5)
3  Period                20.00    0.0626  (5)
4  Subject(Sequence)     20.00    0.0626  (5)


Variance Components, using Adjusted SS

Source               Variance  % of Total     StDev  % of Total
Subject(Sequence)  0.0217111      25.74%  0.147347      50.73%
Error              0.0626500      74.26%  0.250300      86.18%
Total              0.0843611              0.290450
```

Comparisons for Logt½

Dunnett Multiple Comparisons with a Control: Response = Logt½, Term = Treatment

```
Grouping Information Using the Dunnett Method and 90% Confidence

Treatment             N     Mean  Grouping
Zithromax (Control)  24  1.40189  A
Mazit                22  1.38127  A

Means not labeled with the letter A are significantly different from the
control level mean.


Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of      Difference       SE of     Simultaneous              Adjusted
Treatment Levels     of Means  Difference        90% CI       T-Value   P-Value
Mazit - Zithromax     -0.0206      0.0755  (-0.1508, 0.1095)    -0.27     0.787

Individual confidence level = 90.00%
```

General Linear Model: t1/2 versus Treatment, Sequence, Period, Subject

Method

```
Factor coding  (-1, 0, +1)
Rows unused    2
```

Factor Information

```
Factor             Type    Levels  Values
Treatment          Fixed        2  Mazit, Zithromax
Sequence           Fixed        2  AB, BA
Period             Fixed        2  1, 2
Subject(Sequence)  Random      24  2(AB), 3(AB), 5(AB), 10(AB), 11(AB), 12(AB),
13(AB),
                                   14(AB), 15(AB), 16(AB), 20(AB), 23(AB),
1(BA), 4(BA),
                                   6(BA), 7(BA), 8(BA), 9(BA), 17(BA), 18(BA),
19(BA),
                                   21(BA), 22(BA), 24(BA)
```

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Treatment	1	640.1	640.07	0.94	0.344	
Sequence	1	22.9	22.90	0.03	0.867	x
Period	1	740.4	740.44	1.09	0.309	
Subject(Sequence)	22	17563.0	798.32	1.17	0.362	
Error	20	13615.5	680.78			
Total	45	32347.5				

x Not an exact F-test.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
26.0917	57.91%	5.29%	*

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	30.54	3.93	7.76	0.000	
Treatment					
Mazit	-3.81	3.93	-0.97	0.344	1.04
Sequence					
AB	-0.72	3.93	-0.18	0.856	1.05
Period					
1	4.10	3.93	1.04	0.309	1.05
Subject(Sequence)					
2(AB)	-15.2	17.7	-0.86	0.403	*
3(AB)	-16.4	25.0	-0.66	0.520	*
5(AB)	0.4	17.7	0.02	0.982	*
10(AB)	5.7	17.7	0.32	0.753	*
11(AB)	-9.6	17.7	-0.54	0.595	*
12(AB)	3.9	17.7	0.22	0.827	*
13(AB)	24.7	17.7	1.39	0.179	*
14(AB)	46.4	17.7	2.61	0.017	*
15(AB)	-15.9	17.7	-0.90	0.381	*

```
  16(AB)          -12.3     17.7    -0.69    0.496     *
  20(AB)           -3.8     17.7    -0.21    0.832     *
  1(BA)           -22.1     25.0    -0.89    0.386     *
  4(BA)           -24.1     17.7    -1.36    0.189     *
  6(BA)            -4.3     17.7    -0.24    0.810     *
  7(BA)            -0.9     17.7    -0.05    0.960     *
  8(BA)             3.6     17.7     0.21    0.839     *
  9(BA)            -3.1     17.7    -0.18    0.861     *
  17(BA)            9.0     17.7     0.51    0.618     *
  18(BA)           62.7     17.7     3.54    0.002     *
  19(BA)           -8.8     17.7    -0.50    0.624     *
  21(BA)            2.4     17.7     0.14    0.893     *
  22(BA)           -6.3     17.7    -0.36    0.724     *


Regression Equation

t1/2 = 30.54 - 3.81 Treatment_Mazit + 3.81 Treatment_Zithromax
- 0.72 Sequence_AB
       + 0.72 Sequence_BA + 4.10 Period_1 - 4.10 Period_2
- 15.2 Subject(Sequence)_2(AB)
       - 16.4 Subject(Sequence)_3(AB) + 0.4 Subject(Sequence)_5(AB)
       + 5.7 Subject(Sequence)_10(AB) - 9.6 Subject(Sequence)_11(AB)
       + 3.9 Subject(Sequence)_12(AB) + 24.7 Subject(Sequence)_13(AB)
       + 46.4 Subject(Sequence)_14(AB) - 15.9 Subject(Sequence)_15(AB)
       - 12.3 Subject(Sequence)_16(AB) - 3.8 Subject(Sequence)_20(AB)
       - 7.9 Subject(Sequence)_23(AB) - 22.1 Subject(Sequence)_1(BA)
       - 24.1 Subject(Sequence)_4(BA) - 4.3 Subject(Sequence)_6(BA)
       - 0.9 Subject(Sequence)_7(BA) + 3.6 Subject(Sequence)_8(BA)
       - 3.1 Subject(Sequence)_9(BA) + 9.0 Subject(Sequence)_17(BA)
       + 62.7 Subject(Sequence)_18(BA) - 8.8 Subject(Sequence)_19(BA)
       + 2.4 Subject(Sequence)_21(BA) - 6.3 Subject(Sequence)_22(BA)
       - 8.0 Subject(Sequence)_24(BA)

Equation treats random terms as though they are fixed.


Fits and Diagnostics for Unusual Observations

Obs   t1/2    Fit  Resid  Std Resid
 18   13.1   86.1  -73.0      -4.15  R
 25   17.0   17.0   -0.0          *     X
 27   13.2   13.2   -0.0          *     X
 42  174.9  101.9   73.0       4.15  R

R  Large residual
X  Unusual X


Expected Mean Squares, using Adjusted SS

                      Expected Mean Square
   Source             for Each Term
1  Treatment          (5) + Q[1]
2  Sequence           (5) + 1.8333 (4) + Q[2]
3  Period             (5) + Q[3]
4  Subject(Sequence)  (5) + 1.9091 (4)
5  Error              (5)


Error Terms for Tests, using Adjusted SS

   Source             Error DF  Error MS  Synthesis of Error MS
```

```
1  Treatment            20.00  680.7771  (5)
2  Sequence             23.55  793.6517  0.9603 (4) + 0.0397 (5)
3  Period               20.00  680.7771  (5)
4  Subject(Sequence)    20.00  680.7771  (5)


Variance Components, using Adjusted SS

Source             Variance  % of Total    StDev  % of Total
Subject(Sequence)   61.5680       8.29%   7.8465      28.80%
Error              680.777       91.71%  26.0917      95.76%
Total              742.345               27.2460
```

Wilcoxon Signed Rank CI: DiffT-R

```
                                          Confidence
             Estimated    Achieved         Interval
          N     Median  Confidence    Lower   Upper
DiffT-R  24      0.000        90.0   -0.340   0.250
```

Appendix 10: Additional work undertaken using Capillary Electrophoresis (CE) method

OMICS Group Conferences
Accelerating Scientific Discovery

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Analytical techniques for tracking counterfeit and substandard medicines

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Background: Counterfeit and substandard medicines are a global problem affecting both developed and developing countries. Counterfeit medicines are fake, while substandard medicines are true medicines that do not meet the requirements for quality, safety and efficacy of the branded drug. Both counterfeit and substandard drugs can be life threatening and have caused deaths. Thus, it is important to have simple and rapid methodology for detecting counterfeit and substandard medicines. In this study, we used capillary electrophoresis (CE) to detect impurities in lisinopril, high performance liquid chromatography (HPLC) to quantify ciclosporin active ingredients, Ultra Performance Liquid Chromatography with tandem mass spectrometry (UPLC-MS/MS) for detecting impurities in both ciclosporin and azithromycin.

Method: 44 lisinopril, 8 ciclosporin and 19 azithromycin products were obtained from hospitals and pharmacies from 11 countries. In-vitro dissolution testing was used to identify differences between products. It was performed to United States Pharmacopeia (USP) guidelines with sampling at intervals up to 120 min. The samples were quantified by CE, isocratic reverse phase liquid chromatography (RPLC) and UPLC-MS for lisinopril, ciclosporin and azithromycin, respectively.

Results: Impurities were detected in all lisinopril tablets: ranging from 4-27%. For ciclosporin, all capsules met the USP requirements, rupturing within 15 minutes. Statistical analysis showed significant differences ($p<0.0001$) between the mean contents of brand and generic formulations, the confidence interval (CI) 95% range for the brands were (79-108), (71-98), (81-110), (85-117) and (73-100), (83-114), (45-62), for the generics, 95% CI were calculated based on the reference sample being 100%. All brands and two generics showed more than 80% of ciclosporin after 90 minutes (99, 100, 94, 84, 92, 85 and 97%). One generic, contained less than 54% of labelled amount (54±10%). Relative to the brand the mean % content of all capsules were significantly lower ($p<0.0001$). Ciclosporin degradation impurities were detected in one generic preparation.

Conclusion: Based on the results, it is concluded that some of the ciclosporin preparations did not contain the exact mass labelled. Both ciclosporin and lisinopril preparations contained significant impurities. These results have important implications especially with ciclosporin, which has a narrow therapeutic window. Switching among and between branded and generic ciclosporin may lead to irreversible kidney damage or acute rejection.

Biography

Aljohani Badr is originally from Saudi Arabia he received his BSc Pharm from King Saud University in Riyadh and his Master and MPhil degree in Clinical Drug Development from Queen Mary University of London, UK. His professional interests include internal medicine, critical care, pediatrics and clinical research. Now, he is in final year PhD in Clinical Pharmacology, Drug Bioequivalence in QMUL.

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